FY2005 Final Report

Ecological Risk Assessment of Perchlorate In Avian Species, Rodents, Amphibians and Fish

SERDP Project ER-1235

June 2007

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ER-1235: Continuation of the Ecological Risk Assessment of Explosive Residues in Rodents, Reptiles, Amphibians, Fish and Invertebrates: An Integrated Laboratory and Field Investigation Related to Live-Fire Ranges in the Department of Defense

2005 Final Report

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TITLE: Analytical Core

STUDY NUMBER: AS-06-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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RESEARCH INITIATION: January 2006

RESEARCH COMPLETION: December 2006

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb Date
Co-Principal Investigator

_____ _____

Brian Birdwell Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE: Analytical Core for Analysis of Explosives

2.0 STUDY NUMBER:
AS-06-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
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Box 41163
Lubbock, Texas 79409

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 2006
Termination Date: December 2006

6.0 KEY PERSONNEL:

Dr. George Cobb	Co-Principal Investigator
Mr. Jun Liu	Sample preparation, Sample Analysis, and Dose Verification
Ms. Xiaoping Pan	Sample preparation and Sample Analysis
Dr. Ronald Kendall	Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the concentration of explosives and their metabolites in samples from toxicity and environmental fate studies.

8.0 STUDY SUMMARY:
Explosives are widely used within the DOD. Many military installations have soil and/or groundwater contamination problems with these toxicants. We implemented HPLC, GC and LC-MS methods to allow analyte quantification at environmentally relevant concentrations.

9.0 TEST MATERIALS:
Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: 99% purity
 rce: Sigma

RDX

CAS number: 121-82-4
Characterization: 99% purity

MNX
CAS number: 5755-27-1
Characterization: 98% purity

DNX
CAS number: 80251-29-2
Characterization: 72% purity

TNX
CAS number: 13980-04-6
Characterization: 98% purity

HMX
CAS number: 2691-41-0
Characterization: 99% purity

10.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the chronic effects of explosives on wildlife. These data will assist risk assessors in their evaluation of ecological risks at military sites.

11.0 TEST ANIMALS:

None

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Samples were analyzed as submitted from other studies within this research program. Samples were identified by project number, sample type and date.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

We have employed methods developed within previous phases to determine HMX, TNT, RDX, and RDX transformation products in tissues, water, sediment and dosing media. We used LC-MS and GC-ECD techniques for trace quantities, and we used HPLC-UV for dosing media.

This year we processed, prepared and verified dosing solutions for aquatic mesocosm studies and for vertebrate toxicity tests. We have also analyzed several hundred samples for other vertebrate dosing studies. A sizeable amount of our efforts has been to train personnel from other groups and to perform extractions, analyses, and preventative maintenance on instruments.

Our analytical efforts led to publication of six analytically oriented journal articles during the Phase VIII funding cycle. These are attached to this report as pdf files.

1. Pan X, Jones L, Zhang B, TA Anderson, Tian K, Cobb GP. 2006. Liquid chromatography- electrospray ionization-tandem mass spectrometry analysis of octahydro-1,3,5,7- tetranitro-1,3,5,7-tetrazocine (HMX). *Rapid Communications in Mass Spectrometry.* 20 (14): 2222-2226.

2. Pan X, Zhang B, TA Anderson, Cobb GP. 2006. Determination of N-nitroso metabolites of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils by pressurized liquid extraction (PLE) and liquid chromatography-electrospray ionization - mass spectrometry (LC-ESI-MS). J. Chromatog. A., 1107(1-2):2-8.

3. Pan X, Zhang B, Cobb GP. 2005. Extraction and analysis of cyclonite (RDX) and its nitroso-metabolites in animal tissue using gas chromatography with electron capture detection (GC-ECD). *Talanta,* 813-824.

4. Zhang B, X Pan, GP Cobb, and TA Anderson. 2005. Use of pressurized solvent extraction (PSE) /gas chromatography-electron capture detection (GC-ECD) for the determination of biodegradation intermediates of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in soils. *J. Chromatog. B.,* 824:277-282.

5. Pan X, Zhang B, Tian K, Anderson TA, Cobb GP. RDX transformation to N-nitroso compounds in the gastrointestinal tracts of deer mice (Peromyscus maniculatis). *Chemosphere,* 67(6): 1164-1170.

6. Liu J, Severt S, Pan X, Smith P, Cobb GP. 2005. Analysis of HMX in egg extracts using HPLC-MS. *Talanta,* 71(2):627-631.

TITLE: Invertebrate Developmental Toxicity of TNT Metabolites in Soil

STUDY NUMBER: INVDEV-06-01

SPONSOR: Strategic Environmental and Research
Development Program
SERDP Program Office
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RESEARCH INITIATION: January 2006

RESEARCH COMPLETION: December 2006

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Todd A. Anderson Date
Co-Principal Investigator

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Brian Birdwell Date:
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
Invertebrate Developmental Toxicity of TNT Metabolites in Soil

2.0 STUDY NUMBER:
INVDEV-06-01

3.0 SPONSOR:
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SERDP Program Office
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4.0 TESTING FACILITY NAME AND ADDRESS:
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Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start: January 1, 2006
Termination: December 31, 2006

6.0 KEY PERSONNEL:
Dr. Todd Anderson, Co-Principal Investigator / Study Director / Study Advisor
Dr. Andrew Jackson, Co-Investigator
Mr. Baohong Zhang, Co-Investigator
Ms. Christina Freitag, Co-Investigator
Mr. Brian Birdwell, Quality Assurance Manager
Dr. Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The proposed objective was to test the potential impact of TNT metabolites (2A-DNT, 4A-DNT, and 2,4-DNT) on earthworm cocoons and cricket eggs incubated in different soil types (sand, sandy loam, silt loam). For comparative purposes, we also tested the toxicity of the parent explosive (TNT) in the same invertebrate models. These experiments were follow-on studies to those conducted earlier on the biological availability and invertebrate toxicity of explosive (RDX) metabolites in soil and complementary to SERDP-funded toxicity studies on the same compounds to earthworms.

The developmental toxicity of TNT, RDX, HMX, 2A-DNT, 4A-DNT, and 2,4-DNT to earthworm cocoons (*Lumbricus rubellus*) and cricket (*Acheta domesticus*) eggs was evaluated. Because development may be inhibited or halted at contaminant concentrations far below a given LC50, toxicity is often not a useful indicator of the potential ecological consequence of a contaminant (Neuhauser et al., 1985). Therefore,

we assessed reproductive/developmental success (cocoon or egg hatching) upon exposure to TNT, 2A-DNT, 4A-DNT, and 2,4-DNT.

8.0 STUDY SUMMARY:

In these experiments, two different approaches were evaluated to determine the effect of TNT and its metabolites (2A-DNT, 4A-DNT, and 2,4-DNT) on soil invertebrates. The topical and soil incubation tests indicated that TNT and its metabolites have somewhat adverse effects on cricket and earthworm reproductive success, as evidenced by egg and cocoon hatching. However, in most instances, the concentrations at which effects were observed were relatively high. In the cricket assays, the relative toxicities of TNT and its metabolites in general followed the trend of TNT < 2A-DNT < 4A-DNT < 2,4-DNT, although, the toxicity differences were very slight.

In the topical assays with earthworm cocoons, concentrations of TNT, 2A-DNT, and 4A-DNT above 10 ppm adversely affected hatching success. There was also some evidence that these levels slightly delayed the onset of cocoon hatching. In sandy loam soil, 4A-DNT was slightly more toxic to earthworm cocoons than the other test compounds. The relative toxicities of TNT, 2A-DNT, and 2,4-DNT were essentially equal. Interestingly, both RDX and HMX (2 parent explosives) appeared to be slightly stimulatory with respect to earthworm cocoon hatching in sandy loam soil.

9.0 TEST MATERIALS:

Test Material: laboratory sand
Characterization: 100% sand
Source: Fisher Scientific

Test Material: silt loam soil
Characterization: 2.5% organic matter, 34% sand, 54% silt, and 12% clay (pH = 7.0)
Source: Harlan County, NE

Test Material: sandy loam soil
Characterization: 1.3% organic matter, 74% sand, 10% silt, and 16% clay (pH = 8.3)
Source: Terry County, TX

Test Chemical: TNT (trinitrotoluene)
CAS Number: 118-96-7
Characterization: Purity confirmed by source.
Source: Sigma-Aldrich

Test Chemical: 2,4-DNT (2,4-dinitrotoluene)
CAS Number: 121-14-2
Characterization: Purity confirmed by source.
Source: Sigma-Aldrich

Test Chemical: 2A-DNT (2-amino, 4,6-dinitrotoluene)
CAS Number: 35572-78-2

Characterization: Purity confirmed by source.
Source: Sigma-Aldrich

Test Chemical: 4A-DNT (4-amino, 2,6-dinitrotoluene)
CAS Number: 19406-51-0
Characterization: Purity confirmed by source.
Source: Sigma-Aldrich

Test Chemical: RDX (Hexahydro-1,3,5-Trinitro 1,3,5-Triazine)
CAS Number: 121-82-4
Characterization: 99.9% pure
Source: Accurate Energetics, LLC

Test Chemical: HMX (Cyclotetramethylenetetranitramine)
CAS Number: 2691-41-0
Characterization: 99.9% pure
Source: Accurate Energetics, LLC

Reference Chemical: acetonitrile
CAS Number: 75-05-8
Characterization: ACS-Certified
Source: Fisher Scientific

Reference Chemical: deionized water (18MΩ)
CAS Number: NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

While much invertebrate and plant toxicity information has been recently obtained through SERDP-sponsored research on the parent EMs, very little data exist on the potential environmental impact of the degradation metabolites of compounds such as TNT, HMX, and RDX. The products of the biotic and abiotic degradation of these compounds may also pose toxicological risk to terrestrial and aquatic organisms. In some instances, the occurrence of these metabolites in soils at live firing ranges is quite significant (the degradation products of TNT as examples).

The degradation of TNT in soil has been well characterized. Under anaerobic conditions, several metabolites are formed, including 2-amino dinitrotoluene (2A-DNT), 4-amino dinitrotoluene (4A-DNT), and 2,4-dinitrotoluene (2,4-DNT). In addition, these compounds occur frequently in soils at military ranges. Current SERDP-sponsored studies are evaluating the toxicity of the parent compound (TNT) and metabolites (2A-DNT, 4A-DNT, and 2,4-DNT) to soil invertebrates. However, those studies do not include tests on earthworm cocoons or cricket eggs. Our ability to get significant quantities of earthworm (*Lumbricus rubellus*) cocoons through our collaboration with Advanced Biotechnology, Inc. put us in a unique position to test the potential impact of

explosives on soil organisms without duplicating the current SERDP-sponsored efforts. In addition, incorporation of an additional invertebrate model (cricket eggs) (Walton, 1983) allowed us to provide information that is complementary to our previous SERDP-sponsored work.

11.0 TEST ANIMALS:

Species: *Acheta domesticus* (Cricket)
Strain: NA
Age: Eggs
Number: Varies depending on the amount laid
Source: Collected from adult crickets purchased from Carolina Biological Supply Company (Burlington, NC)

Species: *Lumbricus rubellus* (Earthworm)
Strain: N/A
Age: cocoons
Number: Varies depending on the number laid
Source: Advanced Biotechnology, Inc. (Elliott, IL)

Species: *Armadilidium vulgare* (Isopod)
Strain: N/A
Age: Adult
Number: Varies depending on the number required in test
Source: collected locally

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All test systems (crickets, cricket eggs, earthworm cocoons) were placed in bottles with labels containing the appropriate identification information for the test system. Collected samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of controls were used throughout the course of the experiments to ensure the quality of the data generated. Solvent controls (sand or soil amended with acetonitrile only) and negative controls (sand or soil without test compound or solvent) were included in the trials. The solvent controls were prepared in the same manner as toxicant-spiked systems without the toxicant. All data analyses were conducted on nominal metabolite concentrations. Data were processed using standard statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA).

14.0 METHODS:

14.1. Test organisms

Crickets (*Acheta domesticus*) were purchased from Carolina Biological Supply Company (Burlington, NC). They were fed a diet consisting of dog food. Water was supplied daily. Crickets were maintained in aquaria on a 12 h light:12 h dark photoperiod at room

temperature (~20°C). Each aquaria hosted about 15-20 pairs of adult crickets. Cricket eggs were collected as needed for use in experiments.

Earthworm (*Lumbricus rubellus*) cocoons were obtained from Advanced Biotechnology, Inc. (Elliott, IL).

14.2. Cricket egg production
A total of 150 g of fine colored sand (Activa Products Inc., Marshall, TX) was weighed and placed into a 200-mL beaker. Then, 20 mL ultra-pure water was used to wet the sand. Sand was evenly divided into 10 groups and placed into 10 individual 50-mm Petri dishes (VWR International, West Chester, PA). Finally, the sand-filled Petri dishes were put into a 500-mL Redi-Pak straight-sided jar (VWR International, West Chester, PA). Then, two adult female (presence of an ovipositor) crickets were put into the jar and placed in an incubator, overnight, at 28 °C in the dark. After about 12 h of incubation, eggs were harvested for topical tests.

14.3. Topical test: Effect of TNT metabolites on cricket eggs and earthworm cocoons
Two layers of filter paper were placed in 10-cm Petri dishes. Then, 30 cricket eggs harvested from the egg production experiments described earlier were placed on the filter paper. Milli-Q water with different concentrations (0, 1, 10, or 100 μg/mL) of TNT metabolites were added to the exterior of each egg. Three replicates were constructed for each of the exposure concentrations. All treatments were incubated in the dark at 28 °C. Petri dishes were opened each day for observation. The earthworm cocoon experiments were performed in a similar manner.

14.4. Sand test: Effect of TNT metabolites on cricket egg production and hatching
Two healthy adult female crickets were put into a 500-mL glass jar. Each jar contained a 50-mm Petri dish in which 15 g of contaminated sand was contained. Contaminated sand was prepared according to the following procedure. First, 75 g sand was weighed for each treatment. Then, the sand was spiked with 1000 μg/mL of contaminant in acetonitrile (ACN) to a final concentration of 10 μg/g, individually. Spiked sand was thoroughly mixed in order to distribute the contaminant evenly and allow the solvent to evaporate. Spiked sand was further stored for 24 hours in the dark under a chemical hood to permit the complete evaporation of acetonitrile. After 24 hours, the sand was wetted with 10 mL of ultra-pure water (> 18 MΩ). Finally, the spiked sand was evenly divided into five 50-mm Petri dishes (5 replicates). Another 75 g of sand, as control, was treated using the same procedure except that it was spiked only with ACN.

Crickets were kept in the jars overnight (~12 hours), then removed to allow the laid eggs to incubate in the sand in the presence of TNT metabolites. After 45 days, the number of eggs and nymphs was recorded.

14.5. Soil test: Effect of TNT metabolites on cricket eggs and earthworm cocoons
Thirty-five grams of soil was placed into a 50-mL glass jar. Soil was spiked with the test contaminant in acetonitrile to final concentrations of 0, 1, 10, 100, and 300 mg/kg. The spiked soils were mixed thoroughly and placed in a chemical hood overnight to allow the

acetonitrile to evaporate completely. Then, 5 mL of Milli-Q water was added to each jar to moisten the soil. Finally, 20 cricket eggs were put into each jar and covered by a thin layer of spiked soil. Each treatment group contained at least three replicates. After 40 days of exposure, the number of nymphs was recorded. The earthworm cocoon experiments were performed in a similar manner.

14.8. Statistical analysis
All obtained data were statistically analyzed using statistical software (SigmaPlot, Version 8.0, and SigmaStat, Version 2.03, SPSS, Chicago, Illinois, USA). Analysis of variance (ANOVA) was used to compare the data among different treatments. If there was a significant difference among groups or times, LSD multiple comparisons were conducted to compare the mean of each treatment group or time.

15.0 RESULTS:

15.1 Effect of TNT metabolites on cricket egg production and hatching
The presence of TNT metabolites in sand (10 µg/g) did not adversely affect the number of eggs produced by crickets (**Figure 15.1.1**). There was no statistically significant difference ($p = 0.932$) in the number of eggs produced among the treatments (acetonitrile control, TNT, 2A-DNT, 4A-DNT, and 2,4-DNT). Crickets produced approximately 300 eggs in a 12 h period regardless of treatment.

Although the test compounds did not affect the number of eggs produced, there was a statistically significant difference ($p = 0.003$) in the number of eggs that hatched following exposure to the test compounds in sand (10 µg/g). For the control group, 56% of the eggs hatched (**Figure 15.1.2**). Exposure to TNT or its metabolites reduced hatching success to approximately 15%. The affect of the test compounds appeared to be more related to concentration rather than the individual compound as there was no difference ($p > 0.774$) in the hatching success among the treatments (TNT, 2A-DNT, 4A-DNT, and 2,4-DNT) in this assay.

15.2. Topical test: Effect of TNT metabolites on cricket egg hatching
In the topical test, cricket egg hatching decreased as concentrations of TNT or its metabolites increased. A slight dose-response relationship was evident for the hatching of cricket eggs topically exposed to TNT or its metabolites (2A-DNT and 4A-DNT) (**Figure 15.2.1**), although the overall treatment affect was not statistically significant ($p = 0.083$). Without TNT or metabolite exposure, 41 ± 10% of eggs hatched. After 30 days of exposure to 1 µg/mL TNT, 2A-DNT, or 4A-DNT, hatching was 41 ± 11%, 42 ± 2%, and 40 ± 12%, respectively. The high exposure concentration (100 µg/mL) did decrease egg hatching compared to controls. In addition, the effect was slightly greater for the metabolites compared to the parent compound. After 30 days of exposure to 100 µg/mL TNT, 2A-DNT, or 4A-DNT, only 27 ± 9%, 23 ± 12%, and 21 ± 5% of eggs hatched, respectively. Compared with the control, the hatching rates decreased by at least 34% after 30 days of exposure to 100 µg/mL TNT, 2A-DNT, or 4A-DNT.

15.3. Soil test: Effect of TNT metabolites on cricket egg hatching
In the soil test, cricket egg hatching decreased as concentrations of TNT or its metabolites increased. A dose-response relationship was evident for cricket eggs incubated in sandy loam soil beginning at concentrations as low as 1 mg/kg (ppm) (**Figure 15.3.1**); the treatment effect was statistically significant ($p < 0.001$). For the control group (0 mg/kg), 47% ± 5% of eggs hatched. There was no difference between this hatching success and that of the ACN-treated group (45% ± 11%). Compared to the controls, low concentration (1 mg/kg) exposure to TNT or its metabolites did not significantly affect cricket egg hatching, however, TNT significantly affected hatching rates in the groups exposed to 100 mg/kg ($p = 0.003$) and 300 mg/kg ($p < 0.001$). The metabolites (2A-DNT, 4A-DNT, and 2,4-DNT) were slightly more toxic than the parent TNT, significantly affecting egg hatching in sandy loam soil at 10 mg/kg. At the highest concentration tested (300 mg/kg), egg hatching was nearly completely inhibited.

15.4. Topical test: Effect of TNT metabolites on earthworm cocoon hatching
TNT and its metabolites adversely affected cocoon hatching in the topical test (**Figure 15.4.1** and **15.4.2**). After 9 weeks of incubation, about 46% of the cocoons hatched in the control group (without TNT or its metabolites). One ppm TNT or its metabolites did not significantly affect cocoon hatching. However, 10 ppm and 100 ppm of the test compounds reduced cocoon hatching success slightly. Overall, TNT and its metabolites were less toxic than RDX metabolites (MNX and TNX) in this assay; 100 ppm MNX or TNX almost completely inhibited earthworm cocoon hatching (data not shown).

15.5. Soil test: Effect of TNT metabolites on earthworm cocoon hatching
The effect of TNT and its metabolites on earthworm cocoon hatching was also investigated in sandy loam soil. In addition, 2 other parent explosives (HMX and RDX) were tested for comparative purposes. Only data for the sandy loam is presented, as this soil represents a case of maximum bioavailability of the test compounds (Zhang et al., 2006a).

In the first experiment, results indicated that TNT and its metabolites generally affect cocoon hatching in a concentration-dependent manner in sandy loam soil (**Figure 15.5.1**). However, RDX and HMX have little adverse affects on cocoon hatching and may even be slightly stimulatory. In the second test, results for TNT and its metabolites were similar (**Figure 15.5.2**). In addition, the lack of an affect of RDX and HMX on cocoon hatching was again evident.

16.0 DISCUSSION

In these experiments, two different approaches were evaluated to determine the effect of TNT and its metabolites (2A-DNT, 4A-DNT, and 2,4-DNT) on soil invertebrates. The topical and soil incubation tests indicated that TNT and its metabolites have somewhat adverse effects on cricket reproductive success, as evidenced by egg hatching. However, in most instances, the concentrations at which effects were observed were relatively high. This is similar to our work on RDX metabolites (MNX and TNX) with crickets (Zhang et al., 2006c). In the cricket assays, the relative toxicities of TNT and its metabolites in general followed the trend of TNT < 2A-DNT < 4A-DNT < 2,4-DNT although the

toxicity differences were very slight. Crickets display a critical period of teratogen sensitivity and an ability to metabolize xenobiotics during development. In this experiment, although the test compounds inhibited cricket egg hatching under certain conditions, no gross abnormalities in cricket nymphs were observed. This indicates that TNT and its metabolites are not mutagens or teratogens in this assay.

TNT and its metabolites also affected the hatching success and earthworm cocoons. However, in most instances, the concentrations at which effects were observed were relatively high. This is similar to our work on RDX metabolites (MNX and TNX) with earthworms (Zhang et al., 2006b). In the topical assays, concentrations of TNT, 2A-DNT, and 4A-DNT above 10 ppm adversely affected hatching success. There was also some evidence that these levels slightly delayed the onset of cocoon hatching. The soil tests were much more definitive in assessing the adverse affects of TNT and its metabolites. 4A-DNT was slightly more toxic than the other test compounds. The relative toxicities of TNT, 2A-DNT, and 2,4-DNT were essentially equal. Interestingly, both RDX and HMX (2 parent explosives) appeared to be slightly stimulatory with respect to earthworm cocoon hatching in sandy loam soil.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Neuhauser, E.F., C. Loehr, M.R. Malecki, D.l. Milligan, and P.R. Durkin. 1985. The toxicity of selected organic chemicals to the earthworm, *Eisenia fetida*. Journal of Environmental Quality. 14:383-388.

Walton, B.T. 1983. Use of the cricket embryo (*Acheta domesticus*) as an invertebrate teratology model. Fundamental and Applied Toxicology. 3:233-236.

Zhang, B., P.N. Smith, and T.A. Anderson. 2006a. Evaluating the bioavailability of explosive metabolites (MNX and TNX) in soils using passive sampling devices. Journal of Chromatography A. 1101:38-45.

Zhang, B., R.J. Kendall, and T.A. Anderson. 2006b. Toxicity of the explosive metabolites hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) and hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) to the earthworm, *Eisenia fetida*. Chemosphere. 64:86-95.

Zhang, B., C.M. Freitag, J.E. Canas, Q. Cheng, and T.A. Anderson. 2006c. Effects of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites on cricket (*Acheta domesticus*) survival and reproductive success. Environmental Pollution. 144:540-544.

APPENDIX 1

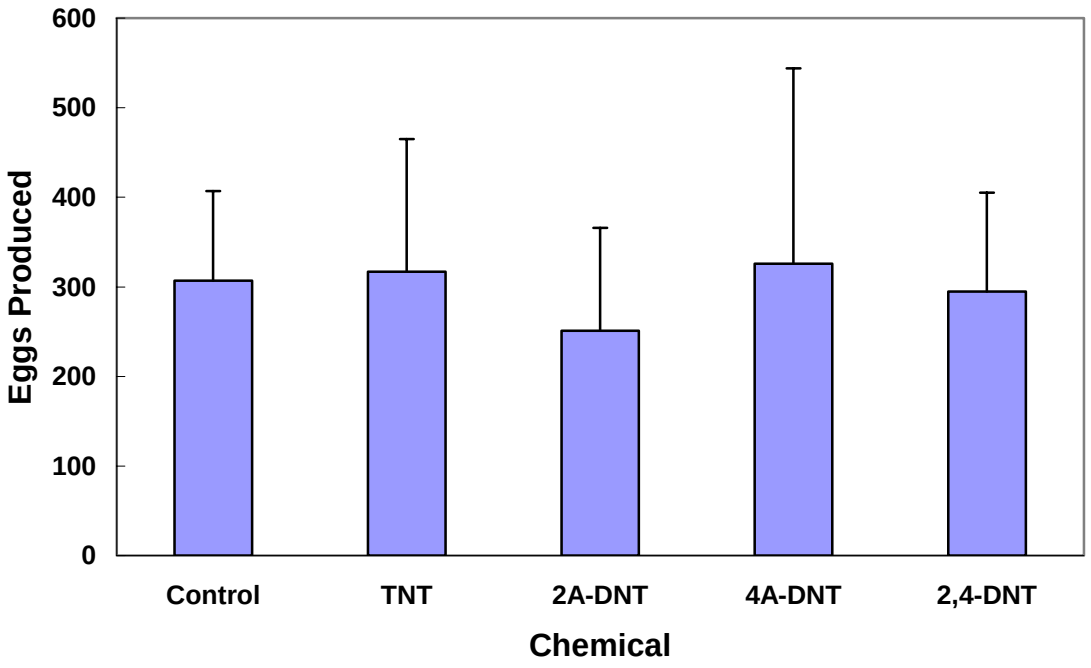

Figure 15.1.1 Effect of TNT and its metabolites (10 µg/g) on cricket egg production in sand. Acetonitrile was used as the solvent control. Error bars indicate one standard deviation of the mean (n = 5).

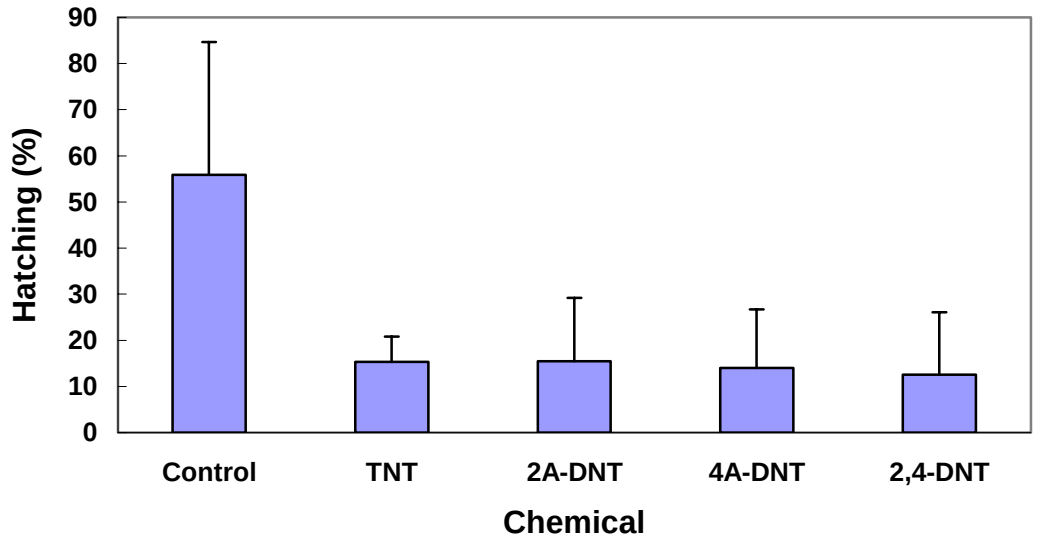

Figure 15.1.2 Effect of TNT and its metabolites (10 µg/g) on cricket egg hatching in sand. Acetonitrile was used as the solvent control. Error bars indicate one standard deviation of the mean (n = 5).

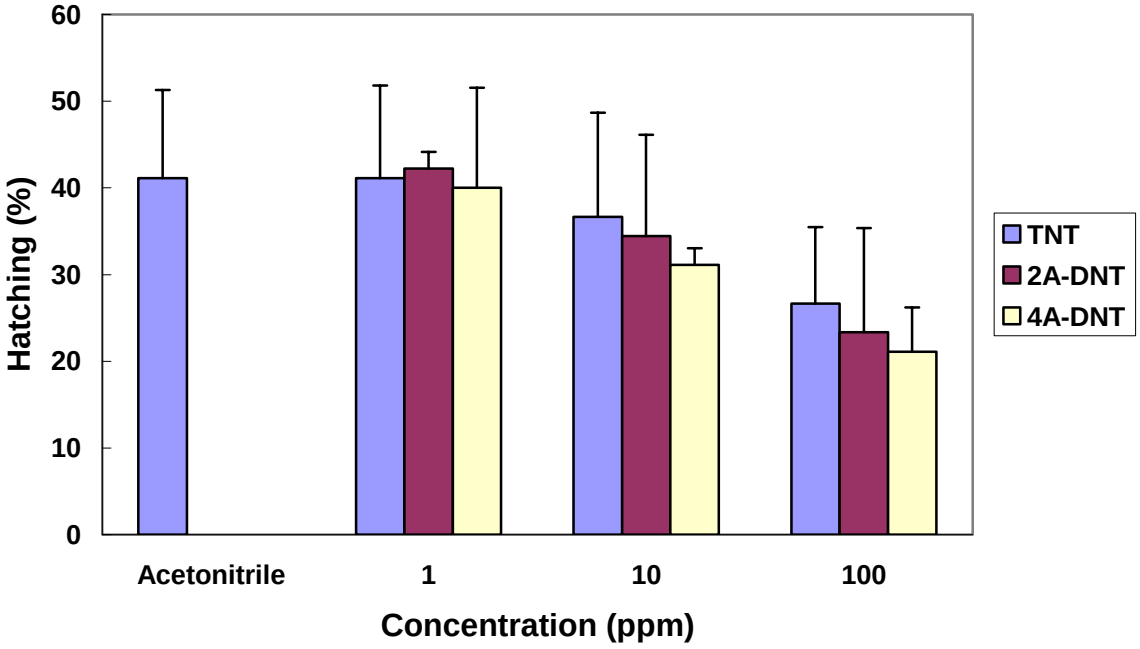

Figure 15.2.1 Effect of TNT and its metabolites on cricket egg hatching in a topical exposure test. Acetonitrile was used as the solvent control. Error bars indicate one standard deviation of the mean (n = 3 replicates). Each replicate contained 30 eggs.

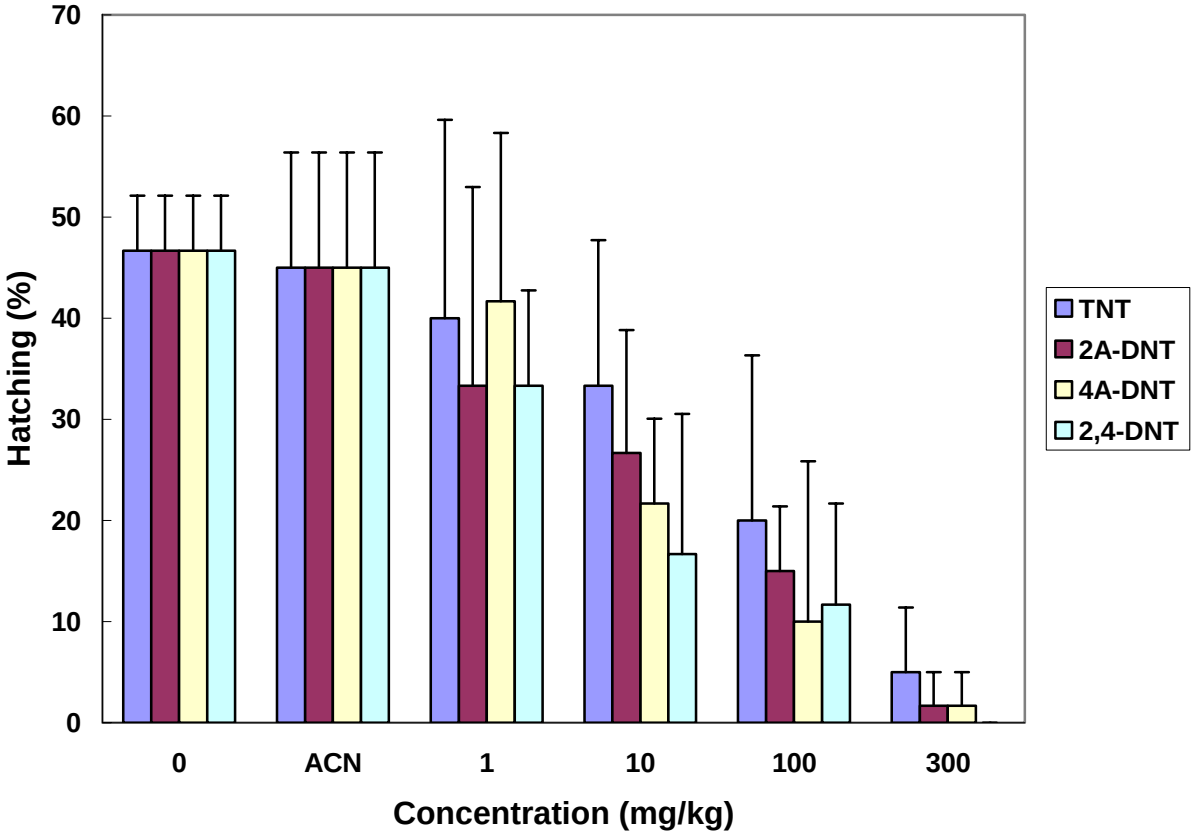

Figure 15.3.1 Effect of TNT and its metabolites on cricket egg hatching in sandy loam soil. Acetonitrile (ACN) was used as the solvent control. Error bars indicate one standard deviation of the mean (n = 4).

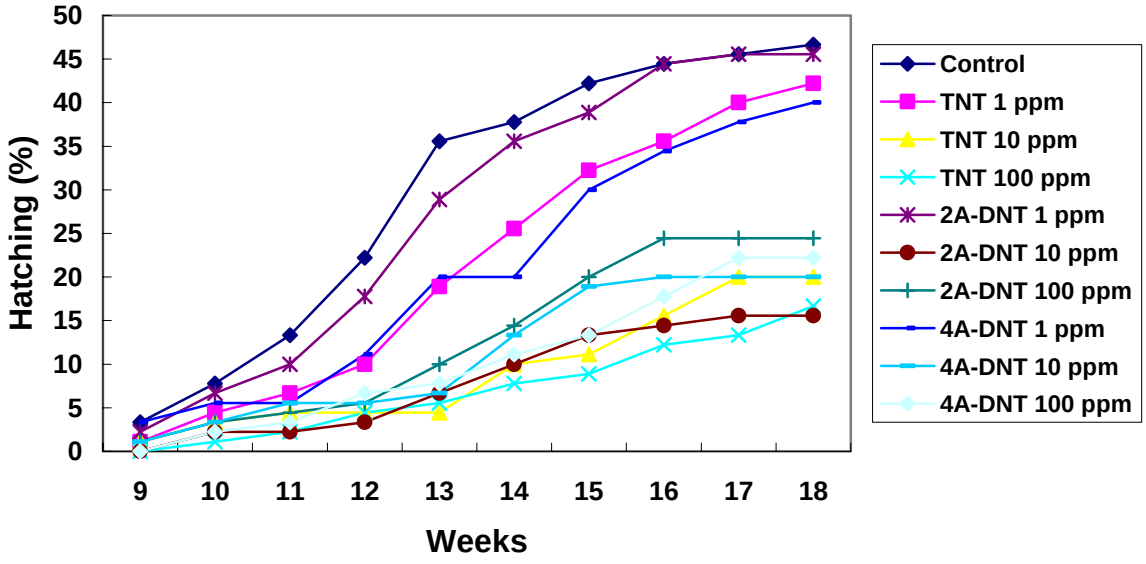

Figure 15.4.1 Effect of topical exposures to TNT and its metabolites on earthworm (*Lumbricus rubellus*) cocoon hatching success.

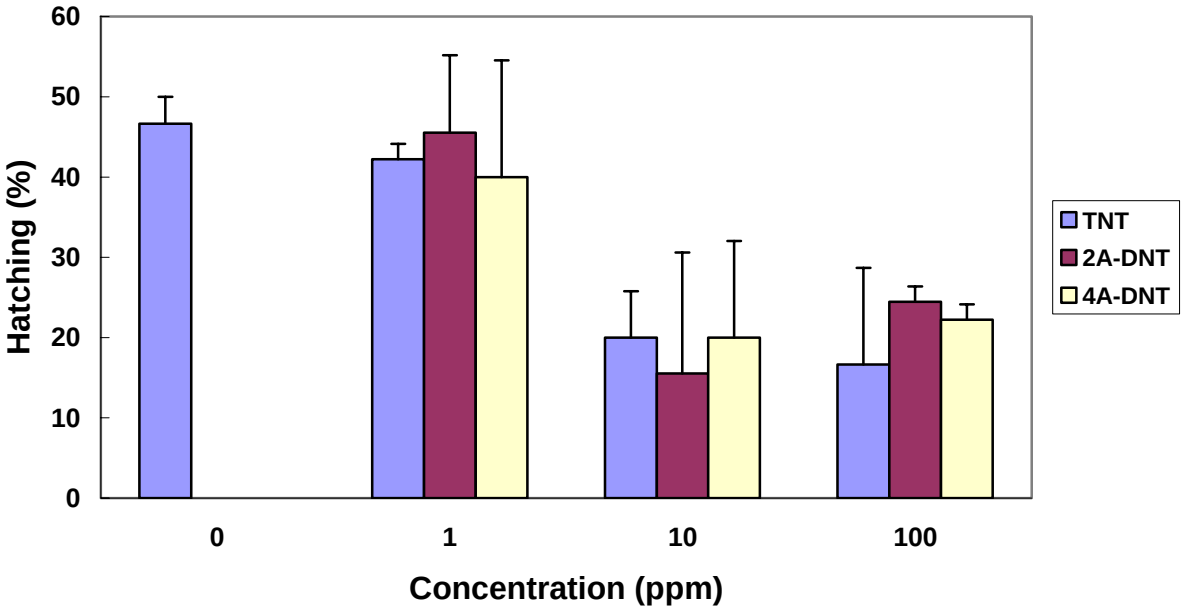

Figure 15.4.2 Effect of topical exposures to TNT and its metabolites on earthworm (*Lumbricus rubellus*) cocoon hatching success at the end of an 18-week incubation period.

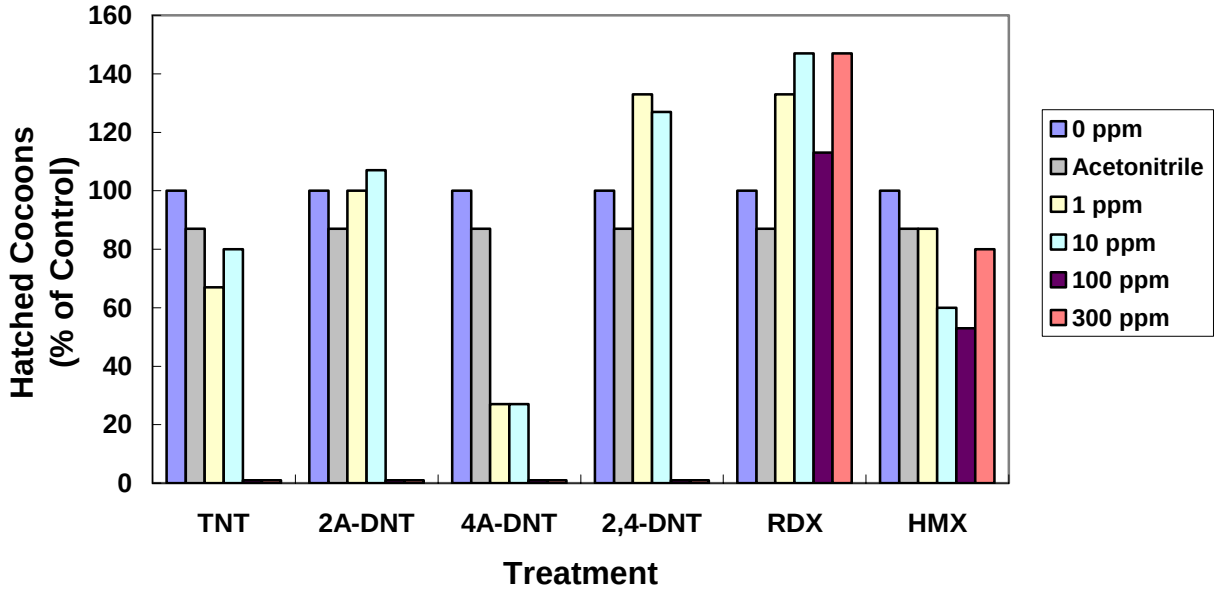

Figure 15.5.1 Effect of TNT and its metabolites in sandy loam soil on earthworm (*Lumbricus rubellus*) cocoon hatching success in Test 1. RDX and HMX were also tested in this assay. Each treatment contained 4 replicates of 20 cocoons.

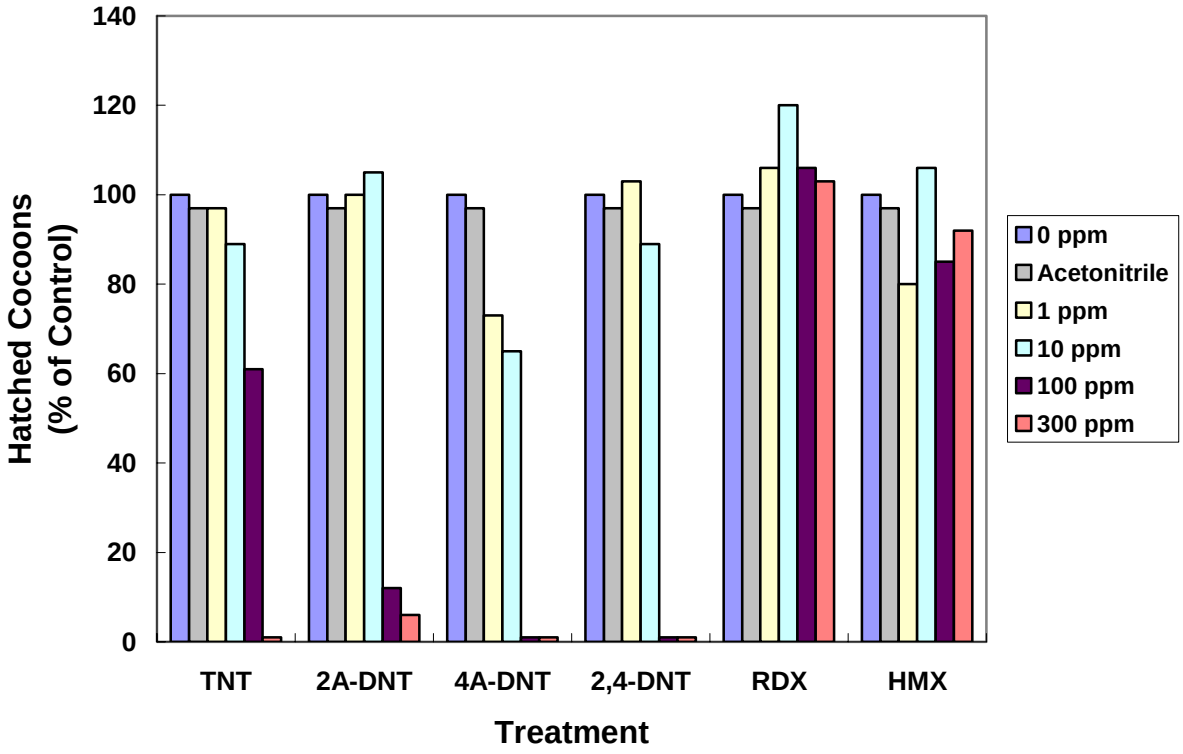

Figure 15.5.2 Effect of TNT and its metabolites in sandy loam soil on earthworm (*Lumbricus rubellus*) cocoon hatching success in Test 2. RDX and HMX were also tested in this assay. Each treatment contained 4 replicates of 20 cocoons.

APPENDIX 2

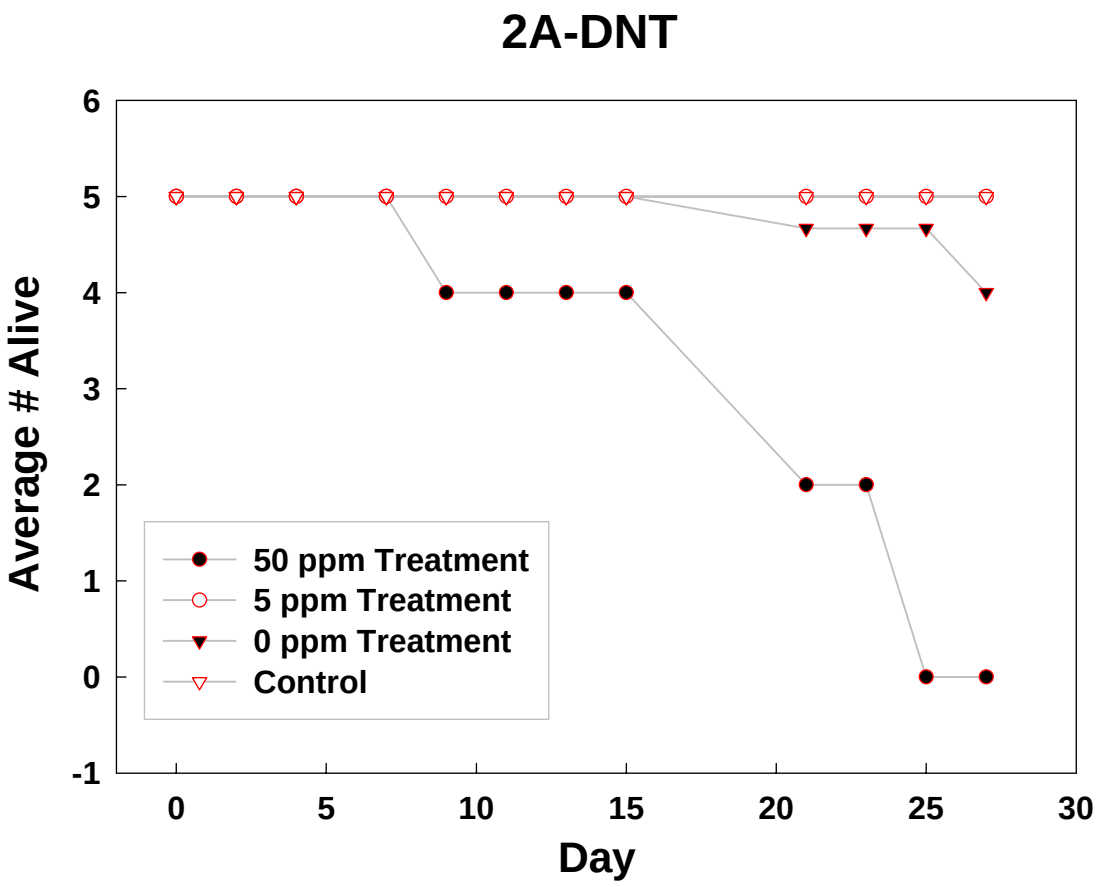

Figure A2.1 Effect of 2A-DNT in sand on isopod (*Armadillidium vulgare*) survival. The control treatment was acetonitrile.

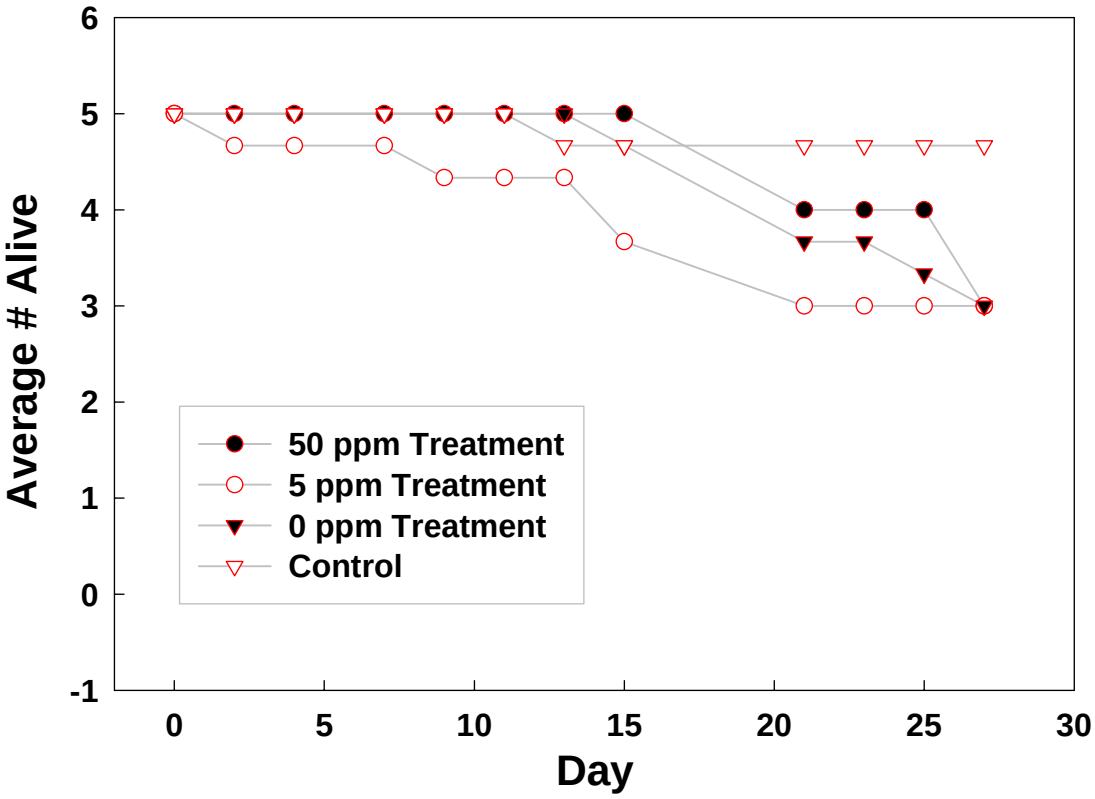

Figure A2.2 Effect of 4A-DNT in sand on isopod (*Armadillidium vulgare*) survival. The control treatment was acetonitrile.

TITLE:	Bioavailability and avoidance of HMX in a terrestrial amphibian
STUDY NUMBER:	HMX-06-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
RESEARCH INITIATION:	January 2006
RESEARCH COMPLETION:	December 2006

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Scott T. McMurry Date
Co-Principal Investigator

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Brian Birdwell Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE: Bioavailability and avoidance of HMX in a terrestrial amphibian

2.0 STUDY NUMBER:
HMX-06-01

3.0 SPONSOR:
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 2006
Termination Date: December 2006

6.0 KEY PERSONNEL:
Dr. Scott T. McMurry, Co-Principal Investigator
Dr. Philip N. Smith, Co-Project Investigator
Dr. Stephen Cox, Co-Project Investigator
Mr. Brian Birdwell, Quality Assurance Manager
Dr. Ronald J. Kendall, Principal Investigator / Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
The first objective of the study was to assess the ability of an amphibian model to discriminate between HMX contaminated soil and uncontaminated soil in a standard choice test, and thereby determine if toads were sensitive to varying concentrations of HMX in the soil. In addition, the second objective was to determine the level of HMX accumulation into toads as a function of dermal exposure during hibernation, to assess the risk of exposure in toads exposed to HMX in the field.

8.0 TEST MATERIALS:

Test Chemical name: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)
95% purity
CAS number: 2691-41-0
Characterization: Explosive
Source: Accurate Energetics

9.0 JUSTIFICATION OF TEST SYSTEM:

The use and subsequent environmental contamination of energetic compounds is an ever increasing international concern (Talmage et al., 1999; USACHPPM 2001; ATSDR 1997). Little is known about the exposure and effects of amphibians exposed to explosives under natural exposure scenarios.

Exposure of terrestrial wildlife species to contaminants can occur through a variety of routes. Dietary exposure is typically considered the primary route, although other routes can play significant roles depending on species-specific life history strategies. For example, species that are intimately associated with the soil, either through their strategy of foraging (e.g., digging in soil for plant or animal food) or using soil as various forms of refugia (e.g., nesting, hibernation, resting) may exhibit above average exposure to contaminants directly from the soil.

Anurans, and true toads (*Bufo* spp.) in particular, can regulate water absorption and loss through a highly vascularized patch of skin called the seat patch, which is located on their posterior ventral surface (Winokur and Hillyard, 1992). In addition, toads appear to be able to "taste" water in soil and determine its suitability for absorption. Evidence for this ability has been demonstrated in toads that could discriminate between suitable and unsuitable levels of urea and sodium chloride in soil water (Brekke et al., 1991; Hoff and Hillyard, 1993). Location of the seat patch and its manipulation by smooth muscle control allow toads to regulate the amount of contact between the seat patch and the ground, thus regulating water absorption and evaporation. This mode of water regulation is thus a likely conduit for absorption of contaminants found in soil water. Recent studies on cadmium exposure in toads have shown that toads can readily accumulate contaminants through their skin during hibernation, and that accumulation is dependent upon soil type and soil moisture (James et al., 2004).

Based on this background information, we propose using toads as an anuran model for assessing bioaccumulation of HMX in a terrestrial environment. Several factors could be addressed with this model, including bioaccumulation of HMX in hibernating and non-hibernating toads (differential accumulation due to temperature and metabolic state of the animal), effects of soil moisture and soil type on HMX bioaccumulation (integrates the level of water regulation in the toad with the solubility of HMX in water), and dose-dependence of HMX bioaccumulation. Results from this study will be useful for risk assessors who can use data on HMX concentrations in the soil, and soil characteristics such as

texture and moisture to estimate bioavailability in terrestrial amphibians that use soil for hibernation and estivation.

We will use the Great Plains toad (*Bufo cognatus*) as our model. This species of toad is found throughout much of the plains states, extending from Montana, east and south through the Dakotas into west Texas and then west through Arizona. Most toads exhibit many similar life histories, chiefly a mostly terrestrial existence, hibernate underground, and regulate water through a seat patch.

10.0 TEST ANIMALS:

Species: Great Plains toads (*Bufo cognatus*)
Strain: Great Plains toads (free-living)
Age: metamorphs (less than 1 year)
Number: 170
Source: collected locally from playa wetlands in Texas

11.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Toads were collected from the field, housed collectively in one 6 ft. diameter holding tank, and then randomly placed as individuals into test tanks for both experiments. Tanks were labeled with appropriate information such as dose level, test compound, ACUC number, and organism.

12.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Avoidance study—toads were randomly placed into respective treatments (0, 1, 10, 100, and 1000 mg/kg HMX in soil). Upon initially introducing each toad into its own tank, the toad was placed in the center of the tank (straddling the two soil choices, either contaminated or not) under a cup, allowed to settle into the new surroundings, and then uncovered. All test chambers were placed on racks in a stratified random pattern.

Hibernation study—toads were randomly placed into treatments, one toad per hibernation chamber. Hibernation chambers were placed on racks in a stratified random pattern, in the cold room at ambient temperature. Temperature was then lowered to 4C over several days to allow acclimation by the toads.

13.0 METHODS

13.1 Animal collection

Great Plains toad (*Bufo cognatus*) metamorphs (ca. 1-2 g) were collected in June 2005 from a single native grassland playa near Silverton, Texas. All toads were housed at TIEHH in 6-foot diameter outdoor tanks with shade cloth. Tanks contained organic potting soil, gravel, and de-chlorinated water. Toads were fed a diet of small crickets and maintained until the initiation of the avoidance study in September 2005 and hibernation study in November 2005.

13.2 Soil preparation (avoidance study)

Five HMX levels were used in the avoidance study (0, 1, 10, 100, and 1000 mg HMX/kg of soil). The solubility of HMX is 1.5mg/100 mL acetone. In order to produce the prescribed doses, an HMX solution of 8.8 mg HMX/600mL acetone was prepared. The solution was then added to 8 kg of a sandy loam soil that was collected near Ropesville, Terry County, TX. The physiochemical properties of the soil were determined by A&L Midwest Laboratories (Omaha, NE) using standard techniques with the results as follows: 74% sand, 10% silt, 16% clay, 1.3% organic matter, and pH = 8.3.

The soil (8 kg) was hand sifted to 2 mm and then mixed with the HMX solution in a commercial cement mixer for approximately three hours. The control soil was mixed with the same proportion of acetone without HMX in order to prevent bias based on the presence or absence of acetone in the soil. The contaminated soil was then brought into the laboratory and placed under the hood to allow for volatilization/evaporation of the acetone and drying of the soil. The hood door was covered with aluminum foil to minimize light exposure and photolysis of the HMX. Once the soil was dry (ca. 36 hours), five samples (3g each) were removed and analyzed for the presence of acetone. Results were negative and soil dilutions began. To determine the proportions for mixing the dosed soil with clean soil, the formula $C_1V1 = C2V2$ (C1 = 1000 mg/kg soil, V1 = 8kg; C2 & V2 are desired concentration and volume) was used. Using the known concentration of 1000 mg/kg, and the dose needed for the lower concentrations, we calculated the amount of 1000 mg/kg soil required to be mixed with clean soil in order to have the concentrations of 1, 10, and 100 mg/kg levels. Dosed soils were mixed using the same commercial cement mixer as above. Concentrations/ratios of clean to dosed soil are found in Table 1. Three-thousand grams of dosed soil were needed for each dose level.

Table 1: Nominal recipes for HMX spiked soils for use in a study to assess avoidance by Great Plains toads (*Bufo cognatus*). Each dose level was mixed with specified amounts of 1000 mg/kg clean, sifted soil for approximately 2.5 hours in a commercial cement mixer. The clean soil was mixed with a proportional amount of acetone in order to alleviate bias based on the presence or absence of acetone.

Amount 1000 mg/kg soil (g)	Clean soil (g)	Total soil (g) & Dose level
300	2700	3000 g of 100 mg/kg
30	2970	3000g of 10 mg/kg
3	2997	3000 g of 1 mg/kg

In order to confirm dose levels of soils, five 2.5 g samples were taken from each dose level and HMX was extracted by Accelerated Solvent Extraction (ASE). ASE cells were rinsed with acetone and a filter placed in the cell. Soil samples were mixed with sodium sulfate to ensure dryness and then loaded into the cells

which were then capped and placed in the ASE apparatus. Collection tubes were labeled and placed under each cell in the extractor. Upon completion of the cycle, the tubes of solvent were removed, diluted, filtered and placed in vials for High performance liquid chromatography (HPLC). For HPLC, standard solutions for HMX at a concentration of 1000 mg/L in acetonitrile were obtained from Supelco. Acetonitrile was from Fisher Scientific. Working standards were prepared in acetonitrile and water (1 to 1) and spanned a concentration range of 50 μg/L to 10,000 μg/L. Seven standards were prepared to calibration. Excellent linearity was achieved with a correlation coefficient of 0.9999 over the concentration span. Each analysis sequence began with injections of at least three continuing calibration standards within the calibration curve range. Single calibration standards were run with every 10-15 samples in the sequence, and responses were averaged into the existing standard curve. If the instrument response of the calibration standards had changed by 10%, then a new standard curve was developed using all calibration standards. Acetonitrile soil extracts were diluted with 1:1 with water and filtered through a 0.2 μm PTFE syringe filter into autosampler vials. Concentrations of HMX were determined by liquid chromatography with a UV detector. The detector was operated at a wavelength of 254 nm. HMX separations were performed with a Supelco C18 column (4.6 x 25 mm, 5μm packing) using a mobile phase of 50% acetonitrile to 50% water. The solvent flow rate was 1 mL/min and the injection volume was 15 μL. The peak of HMX appeared at 5.2 minutes and a single run takes eight minutes. Peak areas were obtained with a HP3390A integrator. Results shown in Table 2.

Table 2. Abbreviated results of soil testing for Avoidance Study with mean and standard error. (ND = not detected).

Sample ID.	Sample wt. (g)	ng/g HMX in Sample	PPM
cont-01	2.09	ND	ND
cont-02	2.07	ND	ND
cont-03	2.00	ND	ND
cont-04	2.01	ND	ND
cont-05	2.01	ND	ND
Mean: NA			
SE: NA			
1000-01	2..01	942.1642	980.3998

1000-02	2.03	873.75	908.5876
1000-03	2.00	1019.375	1060.744
1000-04	2.04	902.5735	939.2024
1000-05	2.04	895.2206	931.511

Mean: 964.097

SE: 26.80

100-01	2.01	97.45025	99.13555
100-02	2.04	96.20098	97.86468
100-03	2.01	96.89055	98.56617
100-04	2.01	97.57463	99.26208
100-05	2.02	94.43069	96.06378

Mean: 98.1745

SE: 0.584

10-01	2.03	9796.798	9.604704
10-02	2.00	10353.13	10.15012
10-03	2.04	10260.42	10.05923
10-04	2.02	9573.02	9.385314
10-05	2.04	10903.8	10.69

Mean: 9.9778

SE: 0.227

01-01	2.02	965.3465	0.965347
01-02	2.05	909.7561	.0909756
01-03	2.03	1221.675	1.221675

01-04	2.01	1199.005	1.19005
01-05	1.99	756.2814	0.756281

Mean: 1.0104

S.E: 0.3816

13.3 Soil preparation (hibernation study)

Soil was prepared for the hibernation study using the same procedure as for the avoidance study except the mixing time. Because of the number of dose levels, the mixing time was decreased to 45 minutes. For the hibernation study there were twelve dose levels (0, 1, 10, 100, 250, 500, 750, 1000, 1250, 1500, 1750, and 2000 ppm). Samples were prepared for testing in the same manner as for the avoidance study. HMX levels were verified, again, using ASE and HPLC methods on three 3g samples from each of the twelve dose levels. The 2.5 g samples which were obtained from the individual hibernation containers were tested for HMX levels. In order to extract the HMX from the soil, each sample was placed in a 20 mL glass vial and 10 ml of acetonitrile (ACN) was added to each sample. All vials were then shaken manually for one minute every ten minutes for two more hours. After mixing, 5mL samples were made from the extracts using two different dilutions depending on the HMX level in the soil. In dose levels ranging from 0 - 100 ppm, 500 μL of the HMX/ACN mixture and 4500 μL of Milli-Q water were mixed in a tube using a vortex mixer. For the 250 - 2000ppm levels, 50 μL of HMX/ACN mixture was mixed with 4950 μL of Milli-Q water. One mL of the dilution was placed in a Uni-prep™ vial which filters the solution as well as preparing it for HPLC. (ASE extraction was not used for testing this group of samples due to the number of samples that needed to be evaluated.) The same HPLC parameters were used as in the avoidance study soil testing. Results of this testing are shown in Table 3.

Table 3. Abbreviated results of soil testing from hibernation study.

Sample ID	Sample Wt. (g)	ng/g Sample	PPM	Mean PPM
con-1	2.5	1518	1.518	1.54
con-2	2.5	1488	1.488	
con-3	2.5	1626	1.626	
1-1	2.5	21080	21.08	21.46

1-2	2.5	23960	23.69	
1-3	2.5	19338	19.338	2.99
10-1	2.5	2900	2.9	
10-2	2.5	2740	2.74	
10-3	2.5	3340	3.34	
100-1	2.5	171000	171	162
100-2	2.5	165000	165	
100-3	2.5	256000	150	
250-1	2.5	326000	256	
250-2	2.5	330000	326	
250-3	2.5	370000	330	
500-1	2.5	488000	370	445.00
500-2	2.5	477000	488	
500-3	2.5	935000	477	
750-1	2.5	818000	935	879.00
750-2	2.5	884000	818	
750-3	2.5	2098000	884	
1000-1	2.5	1262000	2098	1585.33
1000-2	2.5	1396000	1262	
1000-3	2.5	1861000	1396	
1250-1	2.5	1613000	1861	1769.33
1250-2	2.5	1834000	1613	
1250-3	2.5	2231000	1834	

1500-1	2.5	1817000	2231	2045
1500-2	2.5	2087000	1817	
1500-3	2.5	2461000	2087	
1750-1	2.5	2411000	2461	2490.67
1750-2	2.5	2600000	2411	
1750-3	2.5	2725000	2600	
2000-1	2.5	2907000	2725	3000.67
2000-2	2.5	337000	2907	
2000-3	2.5	2725000	3370	

13.4 Avoidance study testing procedures

Clean and spiked soils (ca. 3 cm depth) were placed on opposite ends of plastic containers (29.5 cm x 18 cm x 9.5 cm) and separated by a 3/4 inch diameter dowel rod (18 cm length). Toads were randomly selected, weighed, and one individual placed at the center of each container (10 replicates per treatment). Observations on day 1 consisted of observing the position of the toads (clean side, HMX side) at 15 minute intervals for 4 hours. Observations were continued on days 2-4, toads were observed at 4 hour intervals from 0800 through 2000 h.

13.5 Hibernation study testing procedures

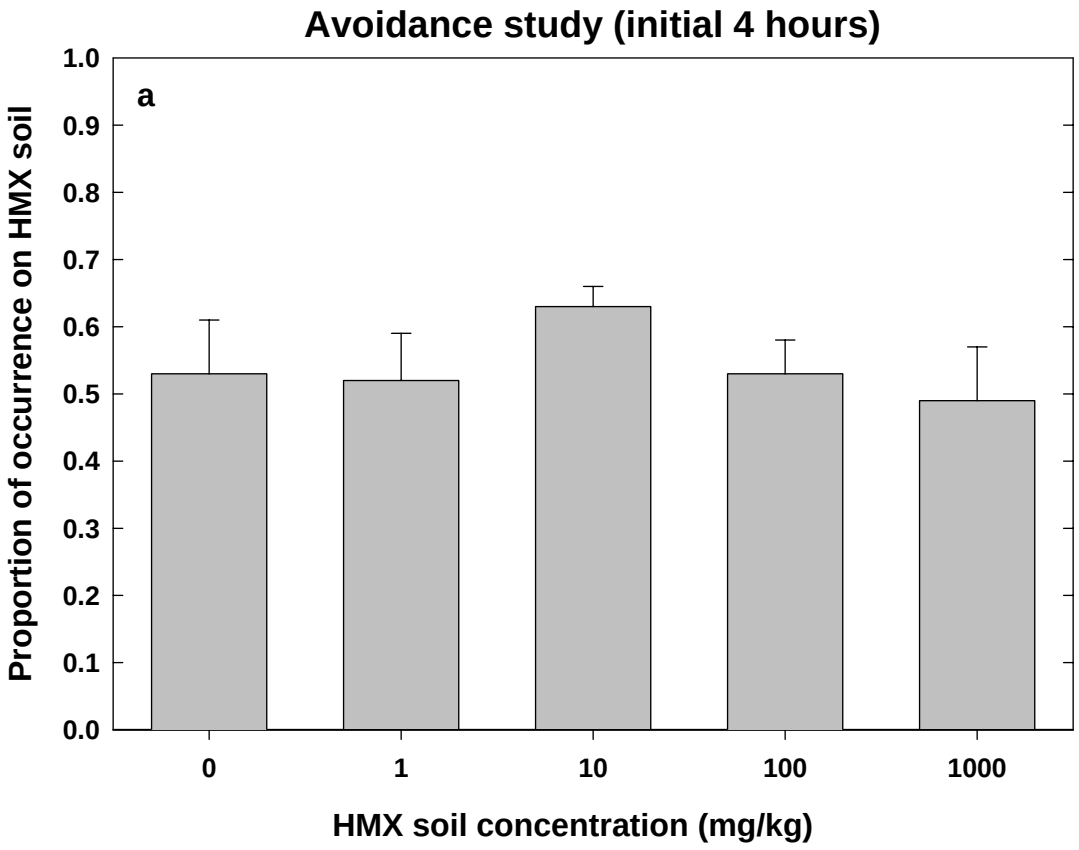
Toads (n = 120; 10 replicates for each dose level x 12 dose levels) were placed in glass hibernation chambers filled with 600 g of soil each and enough water to achieve a moisture level of 21%. Toads were placed in containers in November 2005, the approximate time of the year that they would normally enter hibernation. A 3.5 g sample of soil was taken from each container for verification of the concentration of HMX in the individual container. Toads were weighed and measured prior to placement into containers at room temperature. Containers were weighed with toads, soil and water, and then placed in a cold room and the temperature decreased to 4°C over four days in order to mimic normal seasonal change that triggers burrowing in this species. Containers were weighed weekly from November 2005 until May 2006 and water added to return to the initial weight (moisture level).

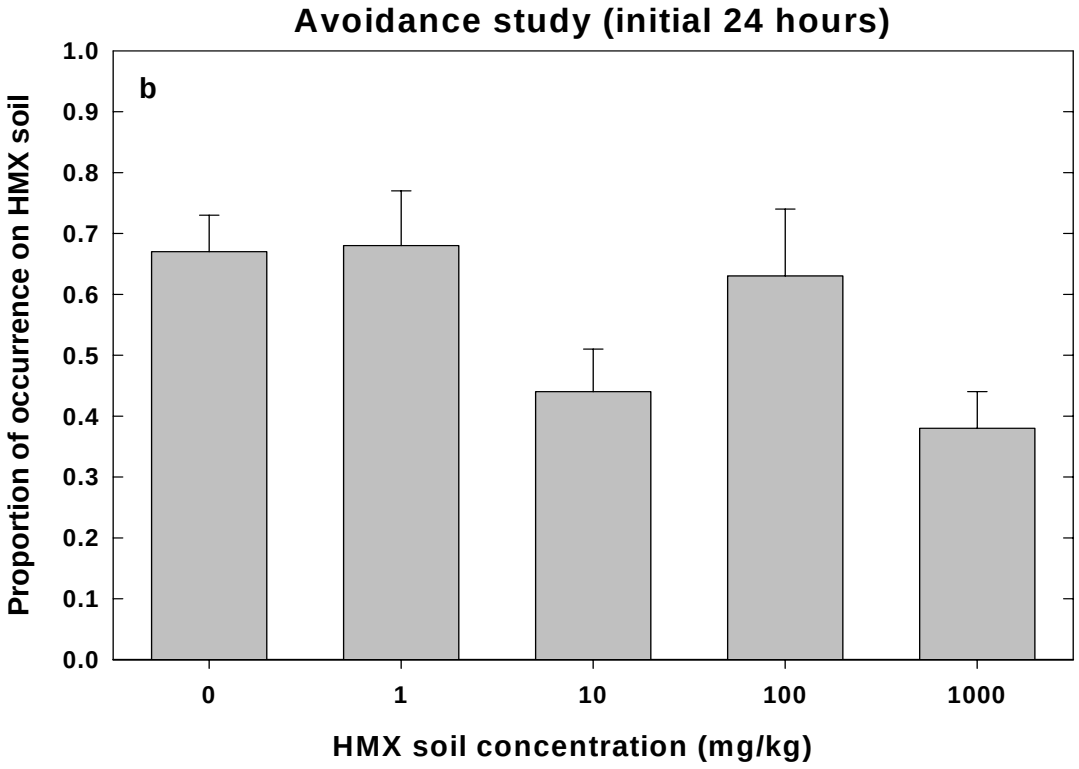
14.0 RESULTS

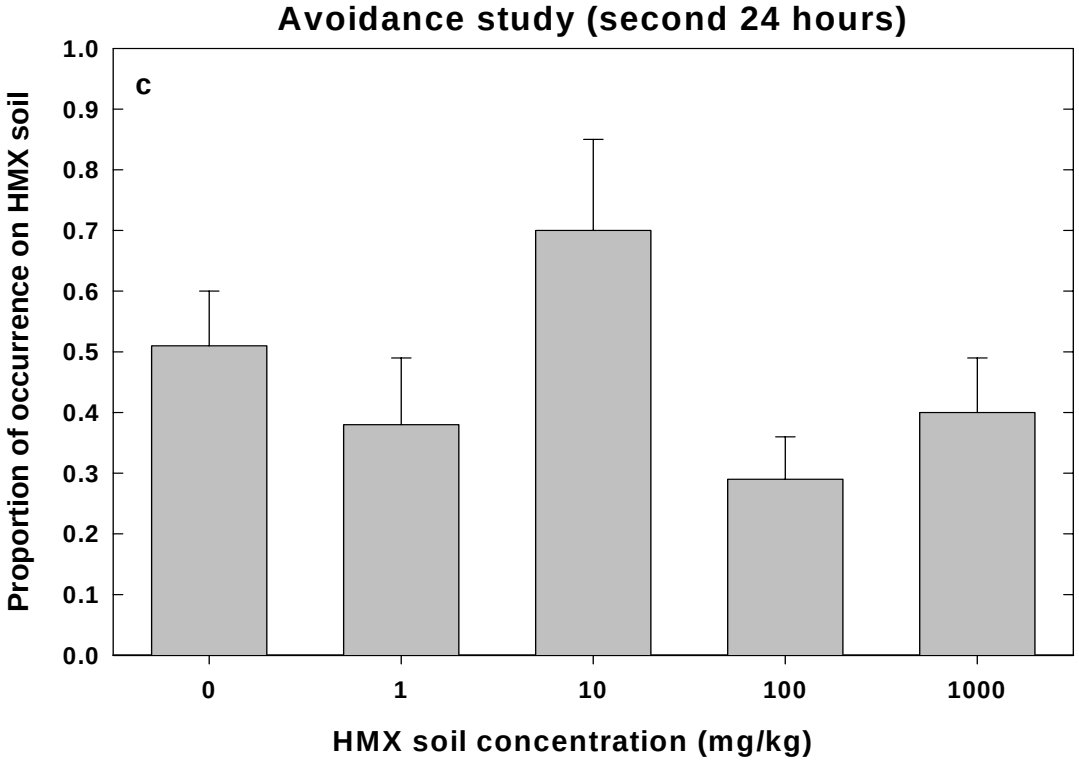
14.1 Avoidance study

Soil contamination levels were confirmed at the prescribed doses and covered four orders of magnitude. Overall, toads showed no discernable avoidance of the

HMX spiked soils during the first 4 h of exposure, with near equal use of the test chamber by all treatment groups (Fig. 1a; P=0.64). Use of the test chamber became more variable however with extended time (Fig. 1b and 1c). There was an indication of non-random use of the test chamber by toads in the 24 h group (Fig. 1b; P=0.03), but not for the subsequent time periods (Fig. 1c and 1d; P= 0.14 and 0.42, respectively). The pattern of use however is nebulous for the 24 h group which, although suggestive of a dose-related effect on which soil toads preferred, is confounded by a suggestion that animals exposed to lower doses (0 and 1 mg/kg) were showing a preference for the HMX side as much as the toads in the 10 and 1000 mg/kg treatments were showing avoidance of HMX soil.




Avoidance study (initial 24 hours)
b
Proportion of occurrence on HMX soil
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
10
100
1000
HMX soil concentration (mg/kg)


Avoidance study (second 24 hours)
c
Proportion of occurrence on HMX soil
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
10
100
1000
HMX soil concentration (mg/kg)

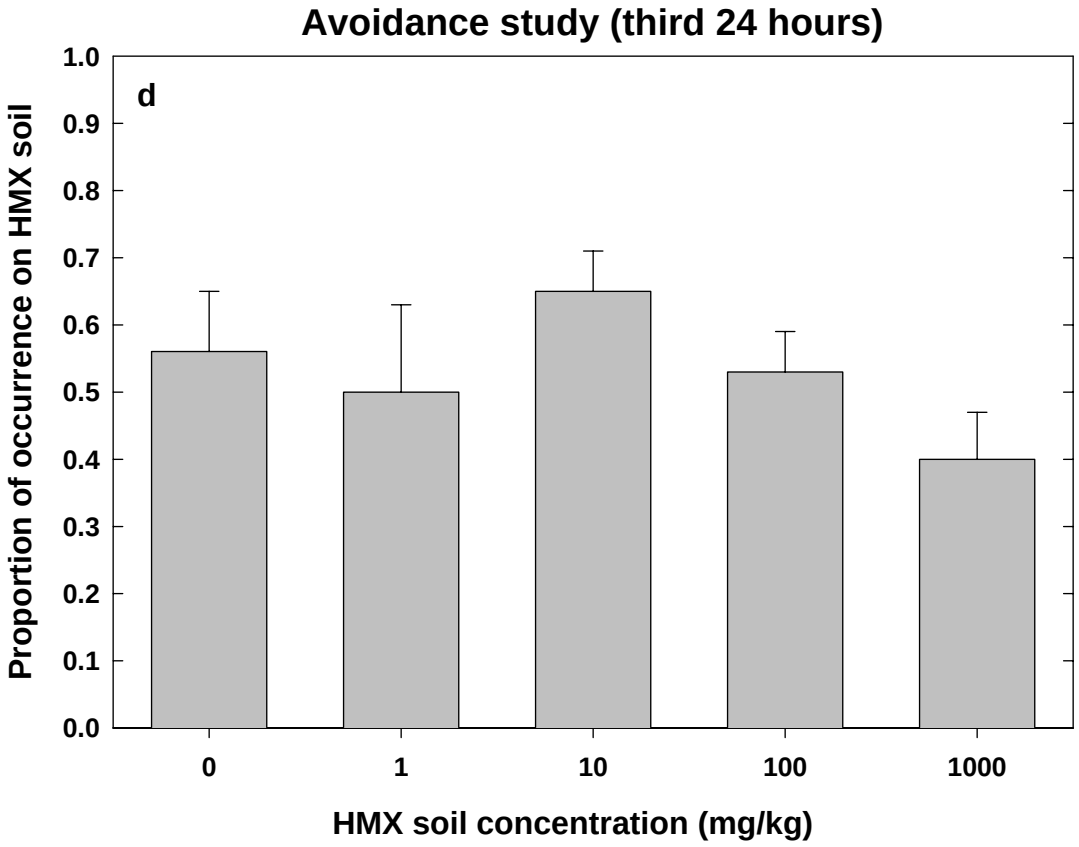


Figure 1. Mean (±SE) proportion of occurrence of Great Plains toads (*Bufo cognatus*) on HMX-contaminated soil versus clean soil during the first 4 h of exposure (a), first 24 h of exposure (b), second 24 h of exposure (c), and third 24 h of exposure (d). Toads were in a test chamber with free choice to move back and forth between HMX contaminated and clean soil. Initial sample size (n) was 10 in all treatments, although some animals escaped during the study, resulting in sample sizes of 10 (1000 mg/kg), 8 (100 mg/kg), 5 (10 mg/kg), 8 (1 mg/g), and 9 (0 mg/kg) by the third 24 h period.

14.2 Hibernation study

All toads except one were dead at the end of the hibernation study. Soil sample testing from both the bulk soil and container samples showed that nominal doses were not achieved. However, HMX doses did encompass four orders of magnitude and this was more important than adhering to exact dose levels (Table 4). Container level results were similar in their deviation from the predetermined contamination levels.

Table 4: Individual container results from hibernation study. Values were not the predetermined levels but covered four orders of magnitude and will serve as an appropriate range of contamination levels.

Nominal level (mg/kg)	Sample Mean (mg/kg)
0	1.99
1	1.86
10	354.76
100	87.88
250	234.88
500	399.16
750	695.66
1000	845.92
1250	1077.82
1500	1546.59
1750	1820.46
2000	2053.11

14.3 Soil residues (avoidance vs. hibernation studies)
Comparing the accuracy of the dose levels of the avoidance and hibernation studies, there are differences in dose accuracy that can be attributed to differences in the mixing protocols (Table 4 shows the mean ppm values for the hibernation containers). Avoidance soils (five levels) were mixed for three hours each and hibernation soils (12 levels) were mixed for only 45 minutes. It was more important that the dose levels be accurate for the avoidance study because there were only four levels. The difference in accuracy in the hibernation soils was not a concern because we achieved an appropriate range of dose levels that would enable the evaluation of the effect of HMX over a four orders of magnitude.

15.0 DISCUSSION

15.1 Avoidance study.
By contaminating soil with various levels of HMX and placing toads on them we are able to examine a toad's ability to discriminate between contaminated and non-contaminated soils. The relatively even use of contaminated and uncontaminated soil by toads in all test groups during the first 4 h likely results from random exploratory movements of toads. Although this pattern changed during extended exposure, there was no convincing indication that toads either avoided or preferred one soil over the other. It is unclear if the lack of effect is due to an inability of toads to recognize HMX in the soil, or if HMX was detected but failed to elicit a negative response. Other species have shown a tendency to

reject food laced with HMX, but even though toads are capable of "tasting" compounds in the soil, this was not evident in this experiment.

15.2 Hibernation study.
The exact cause of mortality of the toads in this experiment is unclear, but is likely do to natural mortality of this particular age group (recently metamorphed) and the extended period of exposure to 4°C, regardless of the fact that this species of toad is known to hibernate at temperatures this low. Larger toads would likely not be as sensitive to these hibernation conditions, but we are unable to acquire larger toads in sufficient number to repeat this exact experiment. Therefore, we decided to conduct this particular portion of the study using a new approach, wherein toads will be exposed to HMX at ambient temperature for 45 days. This approach will still allow us to address the basic issue of HMX accumulation from soil into toads via transfer through the skin. We attempted this approach in summer 2006, using toads purchased from a commercial vendor. However, the animals were diseased and the entire colony had to be euthanized. Therefore, we will collect a new set of *B. cognatus* metamorphs in spring 2007 and finish this phase of our project. Basically, all aspects of this study will remain the same with the exception of the exposure temperature (from 4°C to ambient) and duration (from ca. 6 months to 45 days.).

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TITLE:	Evaluating Metabolic Induction and Reproductive Toxicity of TNT in *Peromyscus Maniculatus*
STUDY NUMBER:	MRT-06-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University/ TTU Health Science Center Box 41163 Lubbock, TX 79409-1163
RESEARCH INITIATION:	January 2006
RESEARCH COMPLETION:	December 2006

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

George P. Cobb Date
Principal Investigator

_____ _____

Brian Birdwell Date
Quality Assurance Manager

1.0 DESCRIPTIVE STUDY TITLE:
Evaluating Metabolic Induction and Reproductive Toxicity of TNT in *Peromyscus Maniculatus.*

2.0 STUDY NUMBER:
MRT-06-01

3.0 SPONSOR:
Strategic Environmental and Research
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SERDP Program Office
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4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 2006
Termination Date: December 2006

6.0 KEY PERSONNEL:

George Cobb	Principal Investigator
Marina Espino	Animal Care and Dosing
Roland Eshiet	Animal Care and Dosing
Jun Liu	Dose preparation and Verification
Les Mc Daniel	Euthanasia
Xiaoping Pan	Animal Care and Euthanasia
Jordan Smith	Animal Care and Euthanasia
Dr. Ronald Kendall	Testing Facility Manager

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the concentration of explosives and their metabolites in rodents following dosing of food containing incurred residues.

8.0 STUDY SUMMARY:
TNT is a widely used explosive within the DOD. Many military installations have soil and/or groundwater contamination problems with this toxicant. We performed chronic reproductive toxicity studies wherein breeding, adult deer mice (*Peromyscus maniculatus*) received drinking water that contained TNT. Dosing of the adults

proceeded throughout breeding, gestation, through weaning.

9.0 TEST MATERIALS:

Test Chemical name: TNT
CAS number: 38082-89-2
Characterization: 98% purity
Source: TNT (VWR)

10.0 JUSTIFICATION OF TEST SYSTEM:

The US Department of Defense desires knowledge regarding the chronic effects of explosives on wildlife. These data will assist risk assessors in their evaluation of ecological risks at military sites.

11.0 TEST ANIMALS:

Species:	*Peromyscus maniculatus*
Age:	Adult; Juvenile 0-45day old
Number:	40 breeding pair plus their offspring
Source:	In house breeding colony that was purchased from the University of South Carolina *Peromyscus* Stock Center
Sex:	Male and Female

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

A Standard Laboratory dosing protocol was used. Animals were housed on a single rack in the dosing room. Each cage was marked with a card identifying the test species, animal identification, toxicant, and dose level.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

13.1 Background

TNT (2,4,6-trinitrotoluene: CAS# 118-96-7) has been widely used in the private sector and in the Department of Defense. TNT contamination at DOD facilities is widespread. TNT is listed as a contaminant of concern at ten NPL sites (www.epa.gov/superfund/sites/query/advquery.htm). According to ATSDR, TNT ranks 81st on the CERCLA Priority List of Hazardous Substances. Although this is not a particularly high ranking, TNT ranks second only to white phosphorous (ranks 19th overall) among ordinance related contaminants. SERDP's list of priorities indicates that no reproductive data exist for mammals exposed to TNT. The ATSDR Toxicological Profile for TNT lists effects from chronic doses (>6 weeks) as: reproductive decreases and alterations of organ weights at doses of 125-200 mg/kg (Dilley et al., 1982, Jiang et al 1991, Levine et al, 1984, 1990).

Animal experiments and epidemiological studies have shown that TNT can induce reproductive toxicity (Homma et al., 2002). TNT administration induced germ cell degeneration, the disappearance of spermatozoa in seminiferous tubules, and a dramatic decrease in the sperm number in both the testis and epididymis. In a human study, significant alterations in male reproductive endpoints have been reported (Li et al. 1993).

Studies in laboratory animals show dose-dependent reproductive toxicity after intermediate oral exposure to TNT. Testicular atrophy, degenerated germinal epithelium, and atrophic seminiferous tubules were reported in male rats after exposure to high doses of TNT (Levine et al. 1984). Evaluation of the toxicity of TNT (1, 5, 25, 125 or 300 mg/kg/day) in Fischer 344 rats for 13 weeks revealed that at 125 mg/kg/day or greater, food intake and body weight gains were reduced, and serum cholesterol levels and anemia increased. In addition, testicular atrophy with degeneration of the seminiferous tubular epithelium was also reported at 125 and 300 mg/kg/day. Short-term oral toxicity of TNT was determined in rats and mice. Single-dose oral LD50s for TNT in corn oil was 1320 and 794 mg/kg in male and female rats, respectively, and 660 mg/kg in both male and female mice. Furthermore, reports of multiple-dose studies, of daily exposure for up to 13 wk with TNT in the diets of rats (0, 0.002, 0.01, 0.05, or 0.25%) and mice (0, 0.001, 0.005, 0.025, or 0.125%) resulted in reproductive effects in the form of reduced testes size in rats at the highest dose regardless of length of exposure and was not reversible following a 4-wk recovery period after treatment. Based on their data Dilley et al., (1982) no-observable-effects-levels for TNT were: rats, 1.42; and mice, 7.76 mg/kg/day. Even so, none of these studies have evaluated the effects that TNT exerts on the number of offspring produced by rodents.

Our study of adverse effects of TNT on the developing organism incorporates continuous parental exposure prior to conception, during prenatal development , and during nursing will allow determination of the time frame within which developmental toxicity is manifested these effects can include: (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) overt functional deficiency. Pertinent endpoints are paternal, maternal and offspring toxicity as outlined by USEPA (USEPA, 1988a; 1988b; 1991) and Kimmel and Price (1990). By evaluating effects on parents and offspring, the data from this project will assist in the risk assessment process concerning the potential developmental and reproductive toxicity of TNT mammalian wildlife. Pre-conceptional (Figure 1) exposures to the parents and *in utero* exposures have been associated with the more commonly examined outcomes (e.g., fetal loss, malformations, birth weight, and measures of infertility). It is anticipated that the minimum evidence necessary to judge that a potential developmental and reproductive hazard of TNT will be demonstrated from this project. Therefore, the specific aim of this sub-project is to evaluate the developmental and reproductive toxicity of TNT in a sentinel mammalian wildlife model- the deer mice. The data from this study will contribute to the ongoing studies as well as regulatory decisions regarding target clean-up levels for TNT.

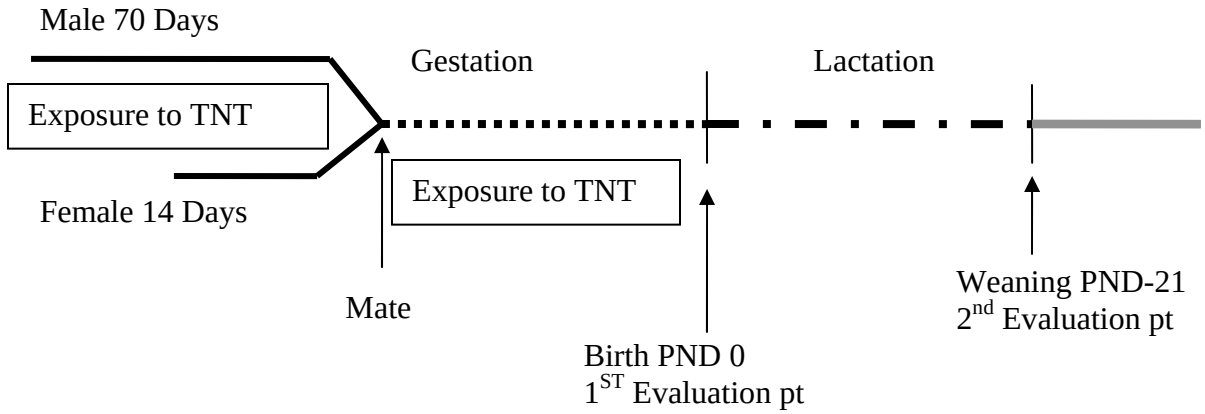

Figure 1. Developmental and Reproductive Exposure and Evaluation of TNT.

14.0 METHODS

Animals: Sexually mature, virgin male and female deer mice (*Peromyscus maniculatus)* were obtained from an in house breeding colony to save costs. Mice were grouped into 10 breeding pair per dose group. These groups received aqueous TNT at the concentrations: control, 10 µg/l, 100 µg/l, and 1000µg/l. All mice were exposed for six weeks before cohabitation to allow male exposure through one spermatogenic cycle.

The study proceeded through cohabitation, gestation, litter delivery, and weaning for each breeding pair (Figure 1, above). The higher dose groups demonstrated delayed reproduction and/or maternal cannibalism of young. Therefore, some pairs were housed through more than one apparent breeding cycle in an attempt to obtain young from each pair.

Chemical Analyses: We determined TNT concentrations in dosing solutions during the course of study. Water was prepared every five days and stored in amber bottles to minimize degradation. Samples from the highest dose were diluted by a factor of 20 before analyses were preformed by LC with UV detection.

Statistical Analyses: All statistical comparisons of pup production and time to birth were done using non-parametric evaluation of ranked data. Significance was ascribed to data that demonstrated $p<0.05$. Chi-square analysis was done to evaluate numbers of litters produced.

Protocols: Animal use and Biosafety Protocols were approved by the Animal Care and Use committee for Texas Tech University.

15.0 RESULTS

Water consumption was quite variable (Table 1). This is likely due to the presence of parents with different numbers of pups until weaning. There were also some cages where mice learned to manipulate the bottles for recreation.

Table 1. Mean water consumption (ml/g/day) of water by breeding deer mice (*Peromyscus maniculatus*) during dosing with aqueous trinitrotoluene

Dose	Water Consumption[a]	Lower CL	Upper CL
Control	0.290	0.087	0.965
10 µg/L TNT	0.342	0.115	1.016
100 µg/L TNT	0.259	0.224	0.298
1000 µg/L TNT	0.268	0.235	0.303

a=Geometric Mean

One male in the high dose group died before cohabitation was initiated. There were no dose dependent differences in live births for the first litter of mice produced by each breeding pair ($P>0.4$). Neither was there a difference in the number of breeding pair whose litters were produced within two weeks of the first litter produced in the study($P>0.4$). There was also not a significant difference in time to birth of the first litter ($p>0.7$). There was, however, a dose dependent decrease in the number of breeding pair that produced a litter of pups within 60 days of cohabitation. There appears to be a dose dependent aggressiveness of males toward their offspring and mates. This resulted in pup mortality in several high dose cages. Overall numbers of live births for two breeding cycles were not different ($p>0.6$) among treatment groups, although there seemed to be a trend of decreased live births in the high dose group. The total number of non-successful pair was one, two, three, in the 10 µg/ml, 100 µg/ml and 1000 µg/ml respective dose groups and then 3 pair in the control group, but this was not significantly different.

Table 2. Numbers of live pups born in the first litter from each breeding pair of deer mice (*Peromyscus maniculatus*) during exposure to aqueous TNT.

	Control	10	100	1000
Pair 1	3	4	3	0
Pair 2	3	2	4	3
Pair 3	4	1	6	0
Pair 4	1	0	0	2
Pair 5	4	2	4	2
Pair 6	1	1	0	1
Pair 7	0	0	0	5
Pair 8	3	3	4	4
Pair 9	4	2	4	0
Pair 10	2	3	2	
Total	**25**	**18**	**27**	**17**

Table 3. Total number of live pups born to each breeding pair of deer mice (*Peromyscus maniculatus*) during exposure to aqueous TNT.

	Control	10	100	1000
Pair 1	5	4	3	0
Pair 2	5	2	4	3
Pair 3	10	5	10	0
Pair 4	1	0	0	9
Pair 5	4	2	9	2
Pair 6	1	3	0	1
Pair 7	0	0	0	5
Pair 8	3	8	4	4
Pair 9	4	2	7	0
Pair 10	2	6	2	
Total	**35**	**32**	**39**	**26**

Significant differences were not found in the number of pups that survived to weaning from the litters that were born first in the first two weeks of litter production ($p=0.21$).

We observed tumors in two adults from the 10 ppb dose group. These were excised at the completion of the study and await histopathology.

16.0 REFERENCES

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TITLE: Assessment of Chronic Exposure to TNT and DNT in a Developing Native Amphibian Species - *Rana catesbeiana*

STUDY NUMBER: RANA-06-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
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CONTRACT ADMINISTRATOR: The Institute of Environmental and Human Health
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ANALYTICAL TEST SITE: The Institute of Environmental and Human Health
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RESEARCH INITIATION: January 2006

RESEARCH COMPLETION: December 2006

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GOOD LABOARATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith
Principal Investigator

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

_____ _____

Brian Birdwell
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE: Toxicity of Chronic Exposure to TNT and DNT in a Developing Native Amphibian Species - *Rana catesbeiana*

2.0 STUDY NUMBER: RANA-06-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
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Texas Tech University / Texas Tech University Health Sciences Center
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 2006
Termination Date: December 2006

6.0 KEY PERSONNEL:
Ernest Smith, Principal Investigator
Norka Paden, Study Director
Ronald Kendall, Testing Facility Management
Brian Birdwell, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:
The specific aim of this sub-project was to evaluate the toxicity of TNT and DNT in a native amphibian model- the American bull frog.

8.0 STUDY SUMMARY:
In summary, this study was designed to determine the effects of TNT and 2,4-DNT, individually, in developing Rana catesbieana larva. To accomplish this objective it was necessary to determine the LD50 values for these compounds. Exposure resulted in abnormalities, these included malformed head, severe optic and thoracic edema, dorsal and lateral flexure of the tail, enlarged intestine, and incomplete coiling and lateral displacement of the intestine. The incomplete coiling and distended gastrointestinal tract observed are likely due to edema as these conditions were seen mainly in larvae that were severely edematous in the abdominal region. While, throphic transfer of TNT is considered to be very unlikely, the direct developmental effects of the parent compound and the biotransformed products requires further evaluation in amphibian species. It is clear that our results suggest that TNT and 2,4-DNT, at the concentrations used in this study, appear to induce developmental toxicity to Rana c. larvae during the early stages of development. These toxic effects following exposure to these chemicals appear to perturb biochemical homeostasis that might be related to critical regulatory pathways in the early developmental

stage. Our preliminary data suggest that TNT and 2,4-DNT are lethal above concentration of 1 ppm and is indicated by survival data (presented below) and the induction of morphological changes during larval development of Rana c. Therefore, it is reasonable to expect that these contaminants affect native amphibian species that would be found at DoD contaminated sites, and requires further characterization and evaluation in such species (Theodorakis, 2004).

9.0 TEST MATERIALS:

Test Chemical name: TNT and DNT
CAS number: 118-96-7 (TNT); 121-14-2 (DNT)
Characterization: Explosive, metabolite
Source: TNT (VWR); DNT ALFA AESAR
Test Medium: Deionized water

Reference Chemical name: FETAX medium was prepared using distilled, carbon filtered water and reagent grade salts (NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM; $MgSO_4$, 0.62 mM).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, FETAX salts were added.

10.0 JUSTIFICATION OF TEST SYSTEM:

Explosives have been observed to be moderately to highly toxic to fresh water organisms (Spain et al., 2000, Tamalge 1999). The DoD has identified 17,660 potentially contaminated sites at 1,877 DoD installations and 6,786 Formerly Used Defense Sites (FUDS). Of these, about 7,000 will require cleanup (EPA 2000). Currently there is lack of information on TNT and DNT long-term toxicity studies on amphibians, reptiles, and birds. Moreover, anurans are on the priority list of research studies on explosives (US Army 2001).

The proposed study will contribute to the understanding of the effects of TNT and DNT exposure on development of *Rana catesbeiana*. Amphibians were used in this study because they are particularly sensitive to contaminants, the effects of which maybe manifested as developmental abnormalities, lethality or other toxic responses that may occur (ASTM, 1998). Recent evidence has also suggested that exposure to contaminants increase amphibian's susceptibility to effects of other environmental agents (Burkhart et al., 1998). Also there has been a worldwide decline in population and a high rate of occurrence of deformities in various species of amphibians (Pechmann et al. 1991; Kavlock 1998).

11.0 TEST ANIMALS:

For the subacute study
Species: American bullfrog, *Rana catesbeiana*
Strain: wild type
Age: adults (4-5 inches snout-vent length)
Number: Approximately 15 for each chemical (30 total)
Source: Rana Ranch Bullfrog Co, Idaho

For the Chronic study
Species: American bullfrog, *Rana catesbeiana*
Strain: wild type
Age: larvae (stage 8-11)
Number: Approximately 210 for each chemical
Source: Rana Ranch Bullfrog Co, Idaho and in house bred animals

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank will be labeled as indicated in the TIEHH SOP AQ-1-14, which includes genus and species name, common name, project name and number, Animal Use Protocol number, the date of exposure, and the name of the person responsible for animal care.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The test system consisted of six treatment groups and one control for each chemical. Each treatment group and the control were replicated twice in the range finding studies and five times in the definitive studies

14.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank were labeled as indicated in the TIEHH SOP AQ-1-14, which includes genus and species name, common name, project name and number, Animal Use Protocol number, the date of exposure, and the name of the person responsible for animal care.

15.0 PROCEDURE FOR DETERMINATION OF THE ACTUAL CONCENTRATION OF THE DOSING SOLUTIONS:

15.1. Nominal Dose preparation

Target concentrations in mg/kg BW were calculated before preparing the dosing solution. 2,4,6 TNT and 2,4 DNT were weighed and dissolved in the calculated volume of PEG. Each solution was stirred for two hours or until the toxicant dissolved, whichever was shorter. Dosing solutions were prepared and verified a few hours before dosing and renewed every three days.

16.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

This study consists of 2 experiments. First, a sub-acute toxicity study in order to determine LD50 for TNT and DNT. Second, a chronic toxicity study for analysis of reproductive changes on different stages of *Rana catesbeiana*. For the sub-acute study 15 adult bullfrogs were exposed to TNT and 15 to DNT following the Updown procedure proposed by the EPA (OECD 2001). Test animals were acclimated for 2 weeks. Animals were administered a single dose of the chemical via oral gavage. Animals were evaluated continuously during the first four hours after exposure and twice a day thereafter for up to 14 days.

For chronic toxicity tests, larvae were acclimated at least 14 days prior exposure to TNT and DNT. Exposure was for 90 days in 2.5 gallon aquaria. The design for TNT and DNT concentrations ranging from 0.0625 to 4 ppm obtained from the 96-hour LD50 (Table 1). When animals showed symptoms of irreversible toxicosis such as gasping, loss of righting

reflex, and disorientation they were removed and euthanized by overdose with Methane Sulfonate (MS-222; 1.5 g/L).

Table 1. Design for *Rana catesbeiana* chronic exposure to TNT. The same design applies to the DNT treatment.

Treatments	Concentration * (ppm)	N	Replicates	Total per treatment
TNT	Control	30	3	30
TNT	0.125	30	3	30
TNT	0.25	30	3	30
TNT	0.5	30	3	30
TNT	1	30	3	30
TNT	2	30	3	30
TNT	4	30	3	30
				210

* The determination of treatment concentrations will follow the methodology of the American Society for Testing Materials (ASTM 1998)

17.0 METHODS:

17.1 Test System acquisition, quarantine, acclimation

Adult *Rana catesbeiana* were purchased from Carolina Biological Supply Company. They were maintained in 30-L glass tanks containing dechlorinated water until exposure, approximately two weeks. Larvae *Rana catesbeiana* were obtained from in house breeding pairs and maintained in 2.5 gallon tanks containing dechlorinated water for at least two weeks before exposure.

17.2 Test Material Application

Rates/concentrations: Rates of concentrations for TNT and DNT sub-acute studies followed the Updown procedure (OECD 2001). The concentrations of TNT and DNT for the chronic exposure study was control, 0.125, 0.25, 0.5, 1, 2 and 4 ppm (Table 1).

Frequency: For the subacute toxicity tests substances were administered through oral gavage (single exposure). For the chronic study exposure was continuous for 90 days.

Route/Method of Application: Adult bullfrogs were exposed to TNT and DNT via oral gavage (single exposure). Metamorphic stages were exposed via dermal, oral, and respiratory exposure in the aquaria medium. *Rana catesbeiana* adults are semi aquatic species. Oral gavages method is very rapid and easy if the animal is held properly. It does not require special restraining devices or anesthesia. The material injected is absorbed slowly (Frederick, 2005). Developing stages of *Rana catesbeiana* are fully aquatic (Lanno 1999 & McDiarmid 1999)

17.3 Test System Observation

Water quality, including salinity, conductivity, pH, dissolved O_2, and ammonia were performed at least once a week. Temperature by means of a surrogate tank was monitored daily. Water samples from each tank and diluted stock solution were removed once per week for analysis of test substances. Tank water samples were collected after the medium was renewed. Dead animals were removed and preserved in 10% Neutral Buffered Formalin (NBF).

17.4 Animal Sacrifice and Sample Collections

For the sub-acute toxicity study no sample collections will be performed. All frogs from each tank were euthanized by immersion in MS-222 (3g/L AQ-1-14).

For the chronic exposure selected organs (gonads, brain, and adrenals) were removed, trimmed and weighed. Half of the tissue collected was frozen for protein expression analysis and the rest for histological analysis. Collection of blood sample was attempted however, due to the small sample volume no analysis was done.

18.0 RESULTS AND DISCUSSION:

Sub-Acute Study:

Acute toxicity of explosive contaminants has not been studied in the bullfrog (*Rana catesbeiana*). The wide distribution of bullfrogs over the United States, and its physiological characteristics make this species especially suitable for toxicology studies. In the present study adult male bullfrogs were acutely exposed via oral gavage to 2,4,6-trinitrotoluene (TNT) and 2,4 dinitrotoluene (DNT) using the Up-and-Down procedure. The estimated starting dose was calculated at 400 mg/kg body weight (BW) for TNT based on previous testing of LD50 on other species. However, for DNT the initial dose was 175 mg/kg BW based on the suggested approach by the Organization for Economic Cooperation and Development (OECD) guideline. Animals were evaluated continuously during the first four hours after exposure and twice a day thereafter for up to 14 days. The results indicate that TNT has a higher degree of toxicity compared to DNT. The calculated LD50s were 1060 mg/kg BW and 1098 mg/kg BW for TNT and DNT, respectively. Symptoms of oral acute exposure to TNT seen in this study included dyspnea, cyanosys, tachypnea, decrease in spontaneous motor activities, somnolence, loss of righting reflex, prostration, tremors, tonic and clonic convulsion, salivation, hypertonia, hypotonia, vomiting, and orange urine excretions. Frogs exposed to DNT displayed similar symptoms to that of TNT with the following exceptions: DNT-exposed animals had no change in urine color and their skin color turned light green. In addition, frogs exposed to DNT lost more weight compared to animals exposed to TNT. High oral doses of both TNT and DNT resulted in gross morphological changes including liver and kidney necrosis (Figures 1A and 1B and 2A and 2B). The symptoms of toxicity were present earlier in TNT-treated frogs compared to DNT-treated ones. Interestingly, toxic responses to DNT and TNT in the current study were similar to those seen in other species treated with toluene. The effects on organ weight are summarized in Tables 1 and 2 below.

Table 2. *Relative Tissue weights (grams) of *Rana catesbeiana* acutely exposed to TNT

Tissues	400 mg/Kg	800 mg/Kg	2000 mg/Kg	800 mg/Kg	2000 mg/Kg	2000 mg/Kg	2000 mg/Kg

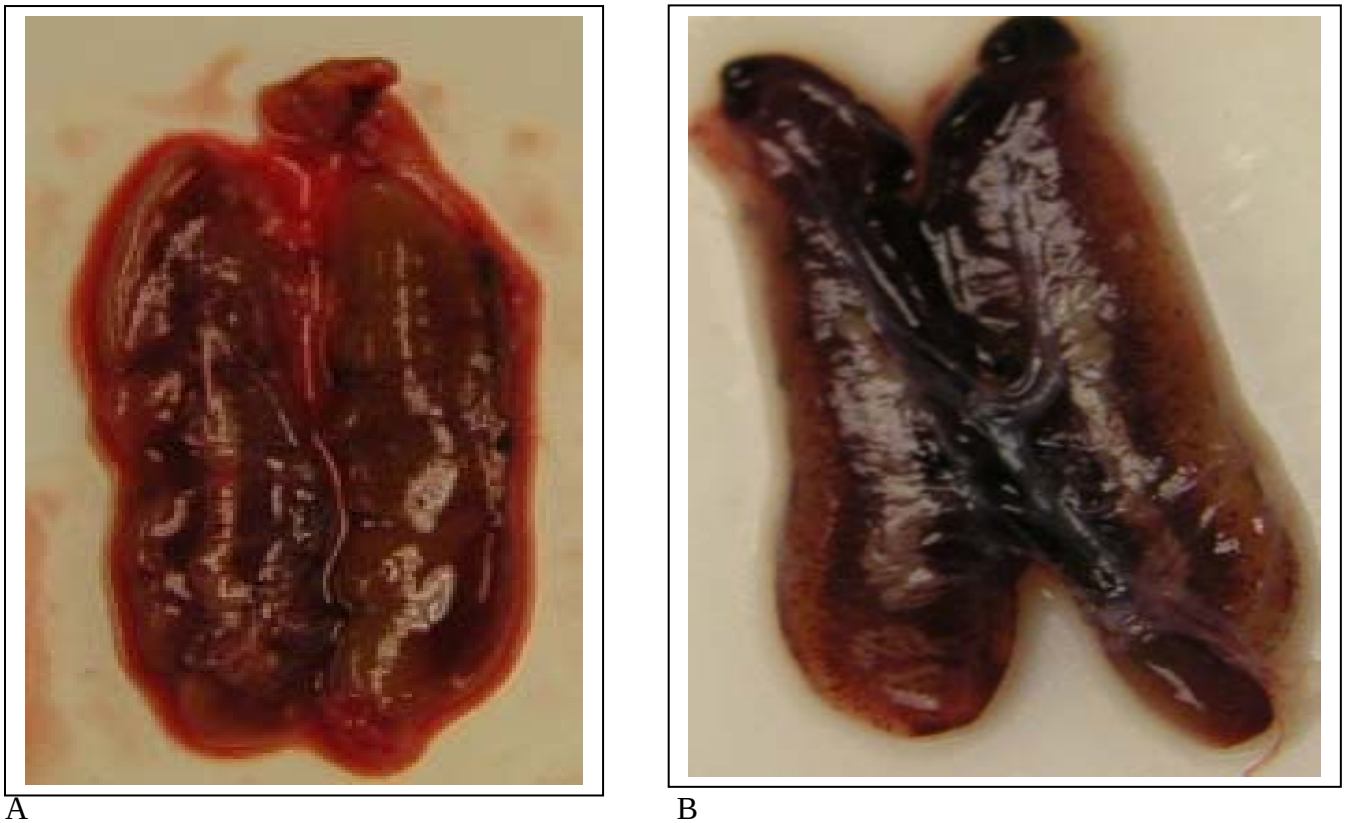
	BW	BW	BW	BW	BW	BW	BW
Liver	1.52	1.97	1.86	2.48	3.86	2.01	3.40
Kidneys	0.41	0.39	0.54	0.36	0.48	0.32	0.40
Spleen	0.13	0.13	0.06	0.06	0.15	0.07	0.08
Heart	0.48	0.40	0.77	0.37	0.46	0.67	0.44

* Percentage compared to the total weight of the animal

Table 3. *Relative Tissue weights (grams) of *Rana catesbeiana* acutely exposed to DNT

Tissues	175 mg/KgBW	500 mg/KgBW	2000 mg/KgBW	2000 mg/KgBW	2000 mg/KgBW
Liver	1.56	1.63	2.27	1.37	1.48
Kidneys	0.50	0.81	0.40	0.50	0.48
Spleen	0.02	0.09	0.14	0.04	0.06
Heart	0.43	0.62	0.33	0.41	0.59

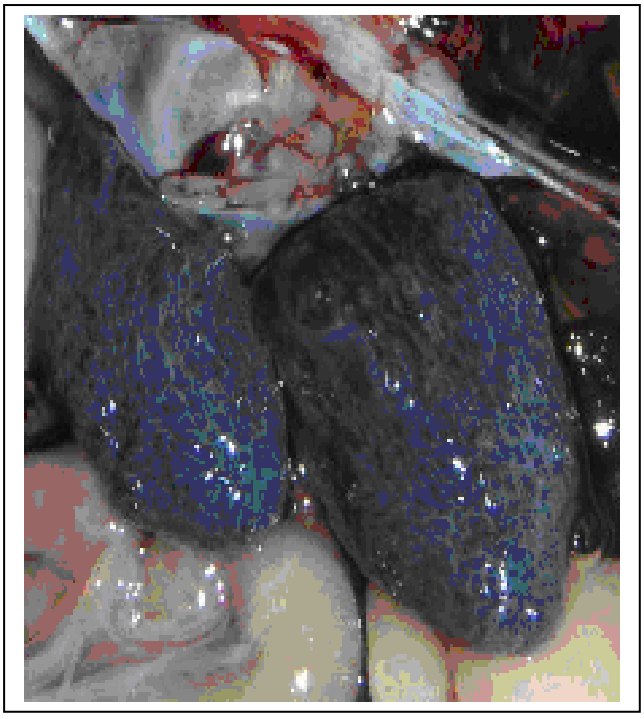
* Percentage compared to the total weight of the animal



A B

Figure 1. A) Photograph of a bullfrog liver following exposure to 175 mg/Kg BW of 2,4-DNT with normal characteristics B) Bullfrog liver following exposure to 2000 mg/Kg BW of 2,4 DNT with signs of necrosis

A

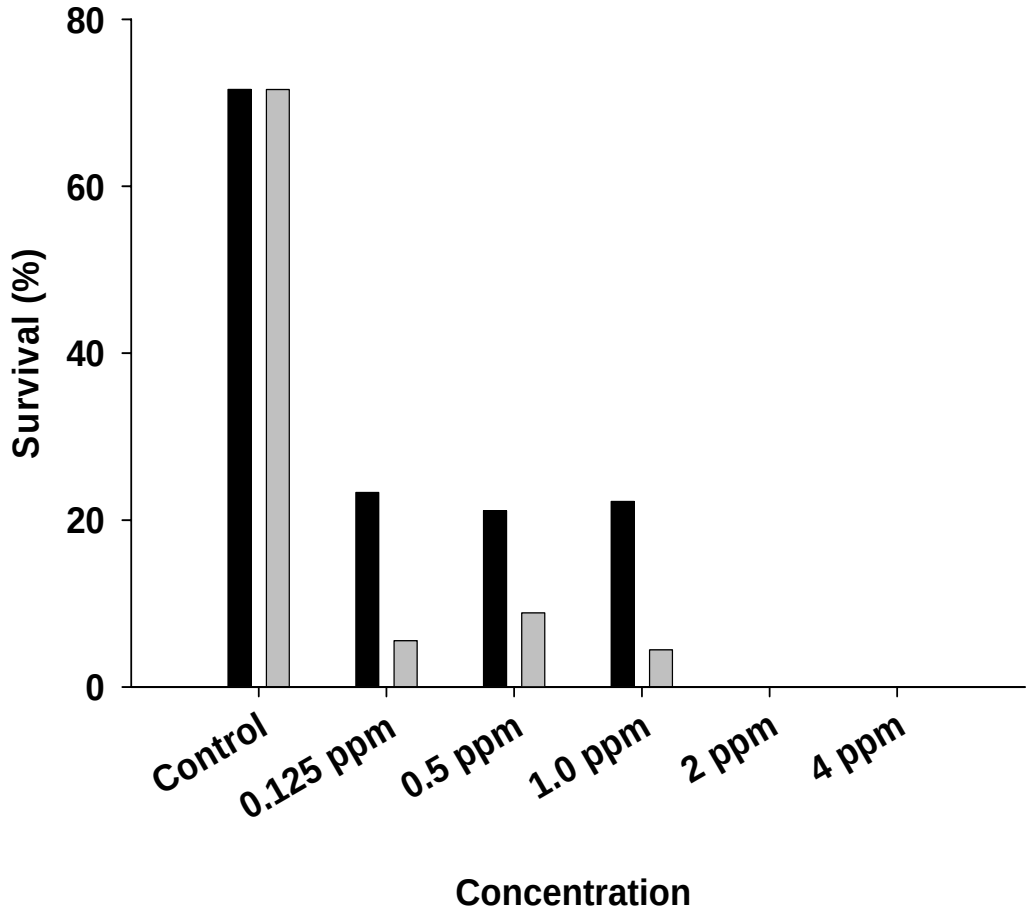


B

Figure 2. Photographs of *Rana catesbeiana* lungs exposed to A) 175 mg/Kg BW of 2,4,6-TNT with normal red coloration B) 2000 mg/Kg BW of 2,4 DNT with a bluish color.

Chronic Study:
Chronic exposure of *Rana c*. to varying concentrations of TNT and 2,4-DNT resulted in significant reduction in the survival of animals exposed during the larval developmental periods. These data are summarized in Figure 3 below for the 12th week of exposure.

Figure 3. Percent Survival of *Rana catesbeiana* following exposure for 12 weeks to either TNT or 2,4-DNT



In addition, chronic exposure of *Rana c*. to varying concentrations of TNT resulted in a slight increase in snout-vent length and body weight after 90 days of exposure during the larval developmental periods. These data are summarized in the Figures 4 and 5 below.

Figure 4. Snout-vent length of *Rana catesbeiana* following exposure varying concentration of TNT (larval stage 24-26)

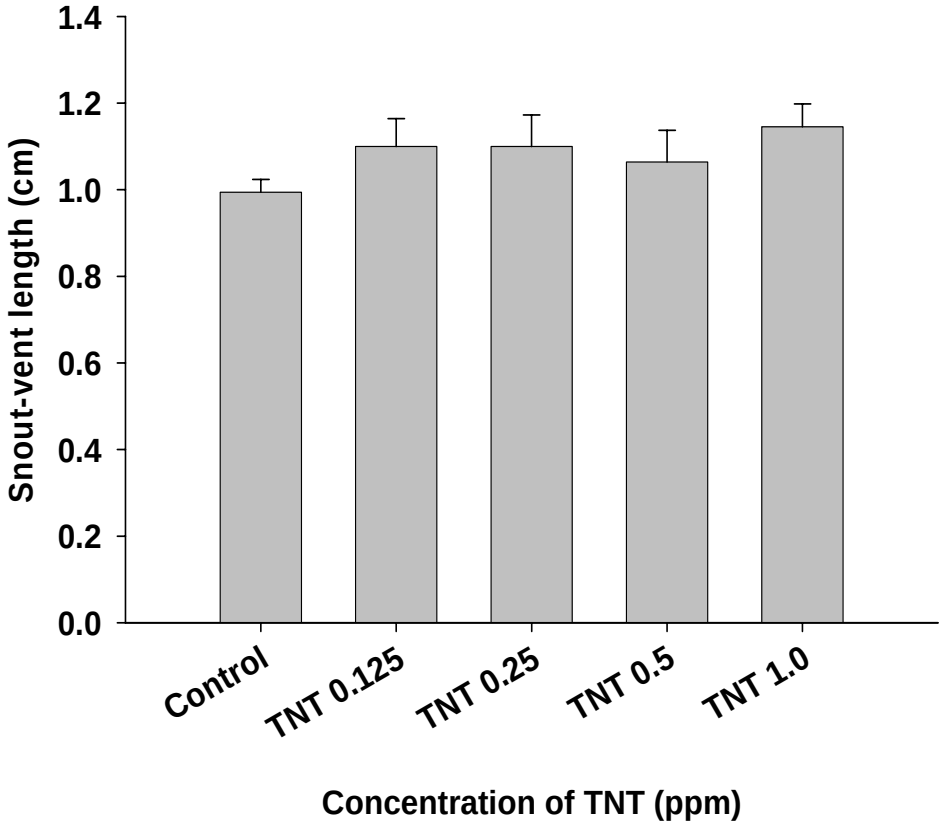
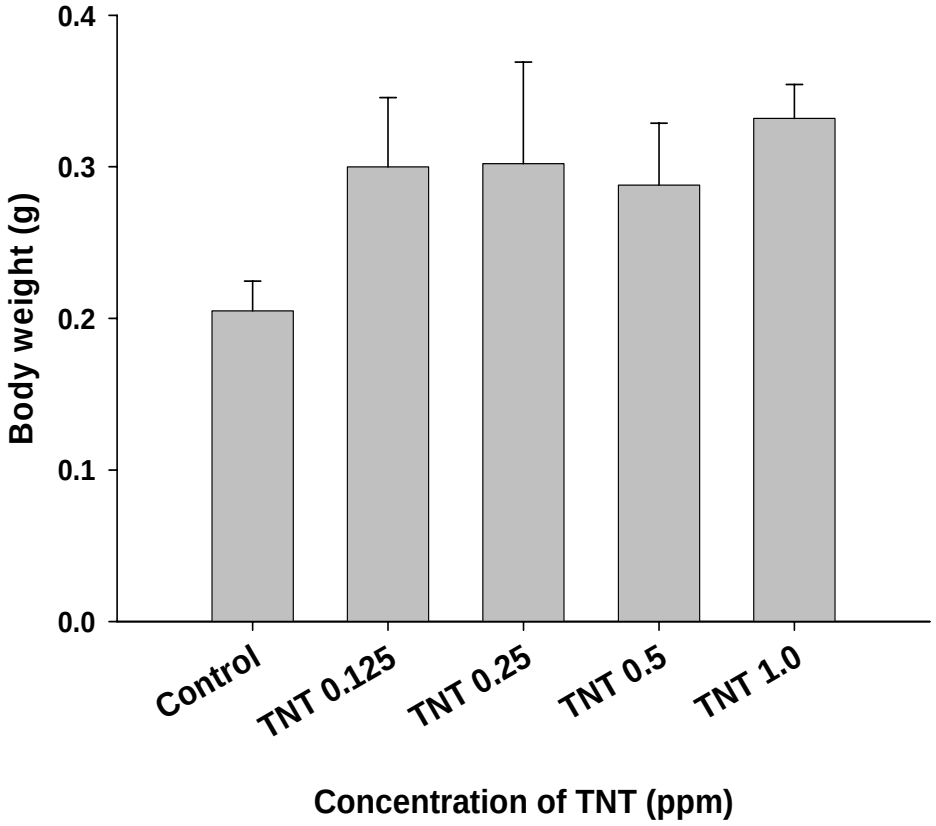
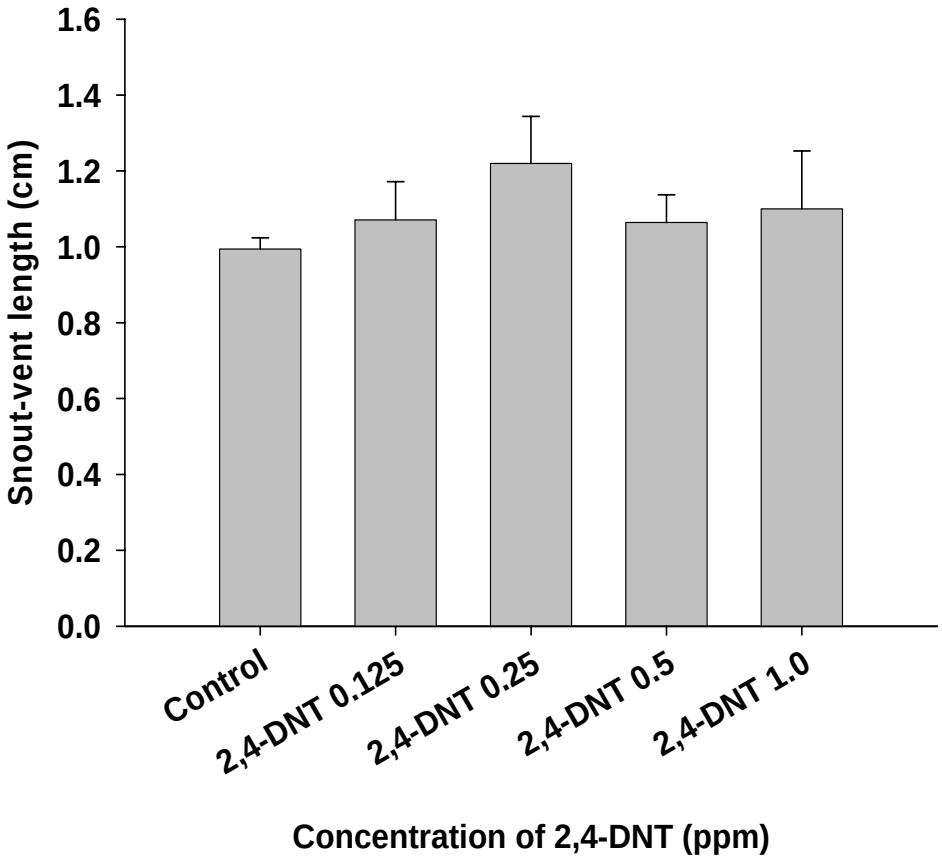


Figure 5. Snout-vent length of *Rana catesbeiana* following exposure varying concentration of TNT (larval stage 24-26)



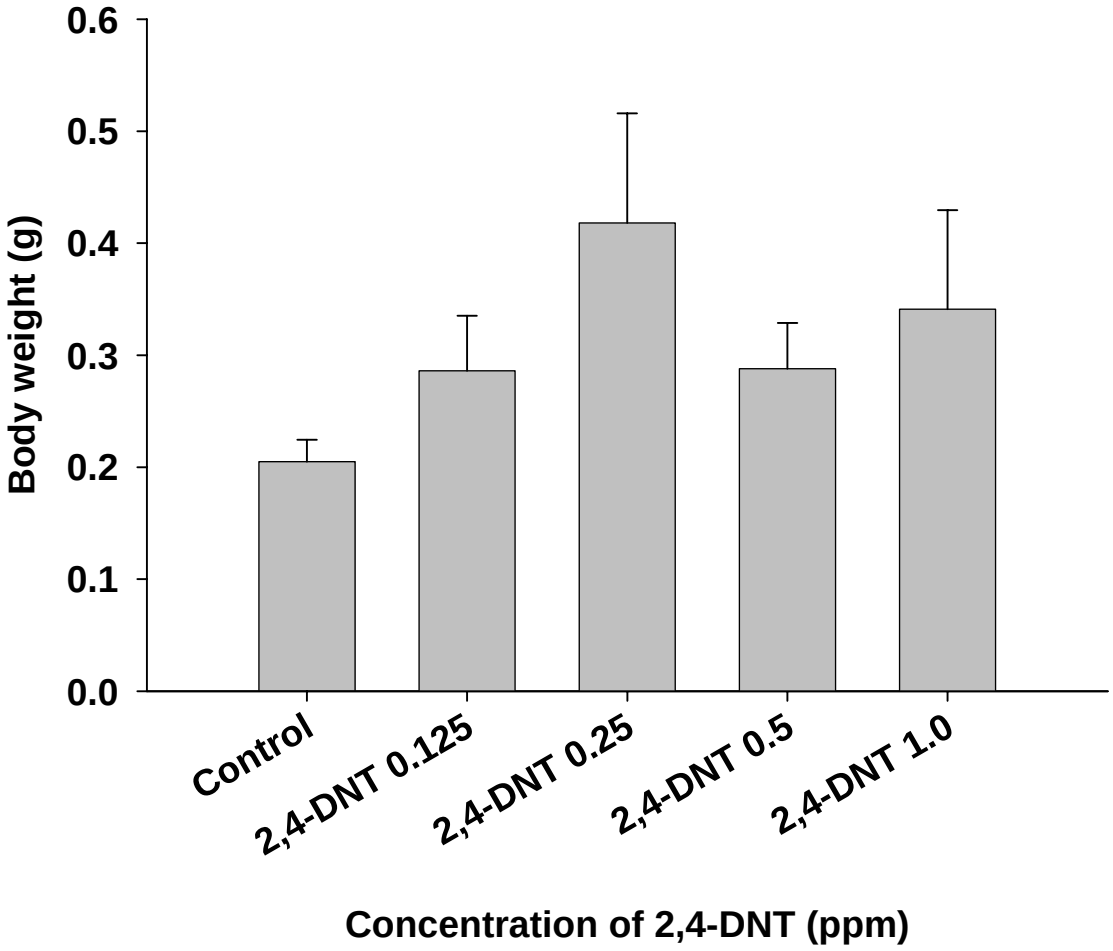
Chronic exposure of *Rana c.* to varying concentrations of 2,4-DNT resulted in a slight increase in snout-vent length after 90 days of exposure during the larval developmental periods. These data are summarized in the Figures 6 below.

Figure 6. Snout-vent length of *Rana catesbeiana* following exposure varying concentration of DNT (larval stage 24-26)



Furthermore, chronic exposure of *Rana c.* to varying concentrations of 2,4-DNT resulted in an increase in body weight after 90 days of exposure during the larval developmental periods. Using Kruskal-Wallis One Way Analysis of Variance on Ranks the differences in the median values among the treatment groups were determined to be greater than would be expected by chance; there is a statistically significant difference ($P = 0.011$). These data are summarized in the Figure 7 below.

Figure 7. Body weight of *Rana catesbeiana* following exposure varying concentration of DNT (larval stage 24-26)



In addition to the gross indication of induced toxicity by TNT and 2,4-DNT to developing Rana c., various indications of morphological abnormalities were observed following exposure to varying concentrations of TNT and 2,4-DNT over an exposure period of 90 days. Representative abnormalities observed during the course of this experiment are included below (Figures 8 and 9 below).

Figure 8. Tadpole exposed to 2 ppm 2,4 DNT with deformed head

Figure 9. Tadpole exposed to 4 ppm 2,4 DNT with curved tail

In summary, this study was designed to determine the effects of TNT and 2,4-DNT individually in developing *Rana catesbieana* larva. To accomplish this objective it was necessary to to determine the LD50 values for these compounds, as reported above. Exposure resulted in abnormalities that included malformed head, severe optic and thoracic edema, dorsal and lateral flexure of the tail, enlarged intestine, and incomplete coiling and lateral displacement of the intestine. The incomplete coiling and distended gastrointestinal tract observed are likely due to edema as these conditions were seen mainly in larvae that were severely edematous in the abdominal region. TNT has been reported to be rapidly transformed and results in minimal accumulation (Belden et al., 2005). But TNT's biotransformation products accumulated to a greater degree than the parent compound. As a result it is not clear if the effects observed in this study are associated with the direct effects of TNT or its biotransformed products. While, throphic transfer of TNT is considered to be very unlikely, the direct developmental effects of the parent compound and the biotransformed products require further evaluation in amphibian species. It is clear that our results suggest that TNT and 2,4-DNT, at the concentrations used in this study, appear to induce developmental toxicity to *Rana c*. larvae during the early stages of development. Toxic effects seen in response to exposure to these chemicals appear to perturb biochemical homeostasis that might be related to critical regulatory pathways in the early developmental stage.

Anuran development is divided into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Dodd and Dodd, 1976; Etkin, 1964). During premetamorphosis, which is the period of embryonic and early larval stage, organogenesis and some advanced morphological changes such as hind limb bud

development occurs. Prometamorphosis is marked with more specific morphogenesis, such as differentiation of the toes and (elongation of the hind limbs). The final period which is metamorphic climax, is associated with metamorphosis, including tail resorption and forelimb development. Our preliminary data suggest that TNT and 2,4-DNT are lethal above 1ppm for TNT and 2,4-DNT, illustrated within the survival data above and the induction of morphological changes during larval development of Rana c. Therefore, it is reasonable to expect that effects of these contaminants can potentially affect native amphibian species that would be found at DoD contaminated sites, and requires further characterization and evaluation in such species (Theodorakis, 2004).

19.0 STUDY RECORDS AND ARCHIVE:

Study records will be maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room temperature and water temperature, salinity, pH, dissolved oxygen content, and ammonia.
- Number of expired animals removed prior to termination of exposure were recorded, including each date and tank.
- Deformities, abnormal swimming behavior and percent metamorphosed animals (complete tail resorption) were recorded daily prior to termination of the experiment.
- Study Methods
- Survival of treatment animals
- Biochemical analysis of results (endpoints mentioned in section 15)
- Interpretation of all data, including statistics results.
- Discussion of the relevance of the findings
- List of all SOPs used.

20.0 RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor in March 2007. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point, upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility for up to 3 years.

21.0 QUALITY ASSURANCE:

The Quality Assurance Unit inspected the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity were brought to the immediate attention of the Study Director. The Quality Assurance Unit periodically submitted written status reports on the study to

management and the Study Director.

22.0 PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, were documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

23.0 REFERENCES:

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TITLE:	Effects of TNT Metabolites on *Xenopus laevis* and *Rana catesbeiana* Larvae
STUDY NUMBER:	TNT-06-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
ANIMAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	January 2006
RESEARCH COMPLETION:	December 2006

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List of Tables and Figures

GOOD LABOARATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in compliance with Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Ernest Smith
Principal Investigator

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Brian Birdwell
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE: Effects of TNT Metabolites on *Xenopus laevis* and *Rana catesbeiana* Larvae

2.0 STUDY NUMBER: TNT-06-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: January 2006
Termination Date: December 2006

6.0 KEY PERSONNEL:
Ernest Smith, Principal Investigator
Mike Wages, Study Director
Ronald Kendall, Testing Facility Management
Brian Birdwell, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:
To determine the acute toxicity and effects of trinitrobenzene (TNB), dinitrobenzene (DNB) and picric acid (PA) on growth and development larval *Xenopus laevis* and *Rana catesbeiana*.

8.0 STUDY SUMMARY:

Xenopus laevis

Xenopus laevis larvae were exposed to TNB, DNB, and picric acid (PA) in separate experiments. The exposure included one control group, 7 concentrations of TNB and PA, and 6 concentrations of DNB. *Xenopus* larvae were exposed to these contaminants starting at Nieuwkoop-Faber (NF) stages 8-10. Exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed larva was counted. Larva found dead before termination of the experiment were removed and placed in 10% buffered formalin.

Rana catesbeiana

Rana catesbeiana larvae were exposed similarly to TNT, HMX, RDX, 2-ADNT, and 4-ADNT in separate experiments. The exposure included one control group and 6 concentrations of each chemical. *Rana* larvae were exposed to these contaminants starting

at Nieuwkoop-Faber (NF) stages 8-10. Exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed larva was counted. Larvae found dead before termination of the experiment were removed and placed in 10% buffered formalin.

9.0 TEST MATERIALS:

Test Chemical name: trinitrobenzene (TNB)
CAS number: 99-35-4
Source: Aldrich Chemical Company

Test Chemical name: 1,3-dinitrobenzene (DNB)
CAS number: 99-65-0
Source: Aldrich Chemical Company

Test Chemical name: Picric acid (PA)
CAS number: 88-89-1
Source: Aldrich Chemical Company

Test Chemical name: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)
CAS number: 2691-41-0
Source: Aldrich Chemical Company

Test Chemical name: hexahydro-1,3,5-trintri-1,3,5-triazine (RDX)
CAS number: 121-82-4
Source: Aldrich Chemical Company

Test Chemical name: 2,4,6-trinitroluene (TNT)
CAS number: 88-89-1
Source: Aldrich Chemical Company

Test Chemical name: 2-amino-4,6-dinitrotoluene (2-ADNT)
CAS number: 35572-78-2
Source: SRI International

Test Chemical name: 4-amino-2,6-dinitrotoluene (4-ADNT)
CAS number: 19406-51-0
Source: SRI International

Xenopus
Reference Chemical name: FETAX medium was prepared using distilled, carbon filtered water and reagent grade salts (NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM; $MgSO_4$, 0.62 mM).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, FETAX salts were added.

Rana
Reference Chemical name: Instant Ocean Sea Salt medium was prepared using distilled, carbon filtered water and 0.36g/L Instant Ocean Sea Salt.
CAS Number: Not applicable
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, Instant Ocean Sea Salt was added.

10.0 JUSTIFICATION OF TEST SYSTEM:

TNT, RDX, HMX, TNB and DNB are synthetic chemicals that are used in explosives. DNB is also used in the manufacture of other products such as dyes and plastics. Picric acid (2,4,6-trinitrophenol) is a chemical that is widely used by the military, in industry, and as a laboratory reagent in research. These chemicals are released in the environment from industrial sources and via discharges from Army ammunitions plants. 2-ADNT and 4-ADNT are metabolites of TNT that are often found in the environment. Characterization of their toxicity is limited and currently, there is no benchmark data for these chemicals in amphibians.

Amphibians were used in this study because they are particularly sensitive to contaminants, the effects of which may be manifested as developmental abnormalities, lethality or other toxic responses that may occur (ASTM, 1999). Recent evidence has also suggested that exposure to contaminants increases amphibian's susceptibility to effects of other environmental agents (Burkhart et al., 1998). Also there has been a worldwide decline in population and a high rate of occurrence of deformities in various species of amphibians (Pechmann et al. 1991, Kavlock 1998).

11.0 TEST ANIMALS:

Species: *Xenopus laevis* (African clawed frog)
Strain: Outbred
Age: embryo/Larvae.
Number: Approximately 1700 (*Xenopus*)
Source: *Xenopus* were bred from captive stocks currently maintained in our laboratory.

Species: *Rana catesbeiana* (American bullfrog)
Strain: wildtype
Age: embryo/Larvae.
Number: Approximately 310
Source: In-house breeding from adults purchased from Rana Ranch.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Glass Petri dishes were labeled with species name, animal use

protocol number, project number, test system, and date of hatch.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The test system consisted of six or seven treatment groups and one control for each chemical. Each treatment group and the control were replicated twice in the range finding studies and five times in the definitive studies.

14.0 METHODS:

14.1 Animal Selection

Adult frogs were selected from in-house breeding colonies. They were selected for breeding if they were not previously bred within the last 90 days.

Assignment of Animals to Study Group and Identification

Larvae were placed into Petri dishes labeled with the name and test chemical concentration, the study protocol number, the animal use protocol number, and a unique identification for each Petri dish. Identification was by test group since identification of individual animals is not possible at this stage of development.

14.2 Acclimation

Xenopus adults were bred in FETAX. *Rana catesbeiana* adults were bred in the Instant Ocean medium used for the study. No acclimation was necessary.

14.3 Animal Husbandry and Test Material Application

Xenopus laevis

Larvae were kept in FETAX solution, specifically formulated for the Xenopus larvae at this stage of development, according to ASTM (1998) or FETAX containing the dissolved test chemical. The FETAX or test solution was changed every 24 hr. The Petri dishes were kept in 50 gal aquarium with water at a level of 4 inches. Plastic grids were used to create a platform on which the Petri dishes were seated. This prevented them from sitting directly in the water. A water heater was kept in the water bath. This maintained the ambient temperature at 23C.

Test solutions consisted of picric acid (75, 100, 125, 150, 175, 200, 225 ppm), TNB (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 ppm), or DNB (6, 10, 14, 18, 22, 24 ppm) dissolved individually in control medium (FETAX). Sufficient amount of each test solution was prepared to last the duration of the experiment. Embryos (Nieuwkoop -Faber [NF] stages 8-10, (Nieuwkoop and Faber 1967) were placed into pre-cleaned Petri dishes containing the appropriate test solution or FETAX. These were allowed to develop for 96 hours while being exposed to the toxicants.

Dishes were cleaned by washing according to SOP AQ-1-23 “Cleaning Glassware for Use with *Xenopus laevis*”, and all Petri dishes were baked at 250° C for 4 hours before use. The overall experimental design consisted of range finding and definitive tests, in which the larvae were exposed to picric acid, DNB, or TNB according to the following scheme:

<u>Range finding tests</u>

There were 10 larvae/replicate x 2 replicates per treatment x 7 treatments per chemical.
Treatment groups consisted of non-treated controls (FETAX) and 6 concentrations picric acid, DNB or TNB.

Definitive tests

There were 10 larvae/replicate x 5 replicates per treatment x 8 treatments for the PA and TNB. The DNB study used 7 treatments.
Treatment groups consisted of non-treated controls (FETAX) and 6 or7 concentrations picric acid, DNB or TNB.

Rana Catesbeiana

Eggs were kept in 20ml Instant Ocean solution (0.36gm/L). The Instant Ocean solution or test solution was changed every 24 hr. The larvae used for 2-ADNT and 4-ADNT were kept in 100ml solution because of their size. The Petri dishes were kept in 50 gal aquarium with water at a level of 4 inches. Plastic grids were used to create a platform on which the Petri dishes were seated. This prevented them from sitting directly in the water. A water heater was kept in the water bath. This maintained the ambient temperature at 23C.

Test solutions consisted of TNT (2.5, 5, 10, 20, 40, 80 ppm), HMX (0.188, 0.375, 0.75, 1.5, 3, 6 ppm), RDX (1.88, 2.88, 5.75, 11.5, 23, 46 ppm), 2-ADNT (1, 2, 4, 6, 8, 10 ppm), and 4-ADNT (1, 2, 4, 6, 8, 10 ppm) dissolved individually in control medium (Instant Ocean solution). Sufficient amount of each test solution was prepared to last the duration of the experiment. Embryos (Nieuwkoop -Faber [NF] stages 8-10, (Nieuwkoop and Faber 1967) were placed into pre-cleaned Petri dishes containing the appropriate test solution or Instant Ocean solution. These were allowed to develop for 96 hours while being exposed to the toxicants.

Dishes were cleaned by washing according to SOP AQ-1-23 “Cleaning Glassware for Use with *Xenopus laevis*”, and all Petri dishes were baked at 250° C for 4 hours before use.
The overall experimental design consisted of range finding tests, in which the larvae were exposed to TNT, HMX, RDX, 2-ADNT and 4-ADNT. According to the following scheme:

Range finding tests

There were 10 eggs/replicate x 2 replicates per treatment x 7 treatments per chemical. (Larvae were used in the case of 2-ADNT and 4-ADNT.)
Treatment groups consisted of non-treated controls (Instant Ocean solution) and 6 concentrations TNT, HMX, RDX, 2-ADNT and 4-ADNT.

Definitive tests

There were no definitive tests. We were unable to obtain more eggs or larvae.

14.4 Daily Observations

All Petri dishes were examined for dead and malformed embryos each day.

14.5 Euthanasia

At the end of the exposure, all animals were euthanized by immersion in 0.5 g/L MS222.

14.6 Sample Collection

Tadpoles were collected at the end of exposure. Endpoints collected were mortality, stage, snout-vent length, and deformities.

15.0 RESULTS:

Xenopus laevis

Experiment I: Exposure to DNB

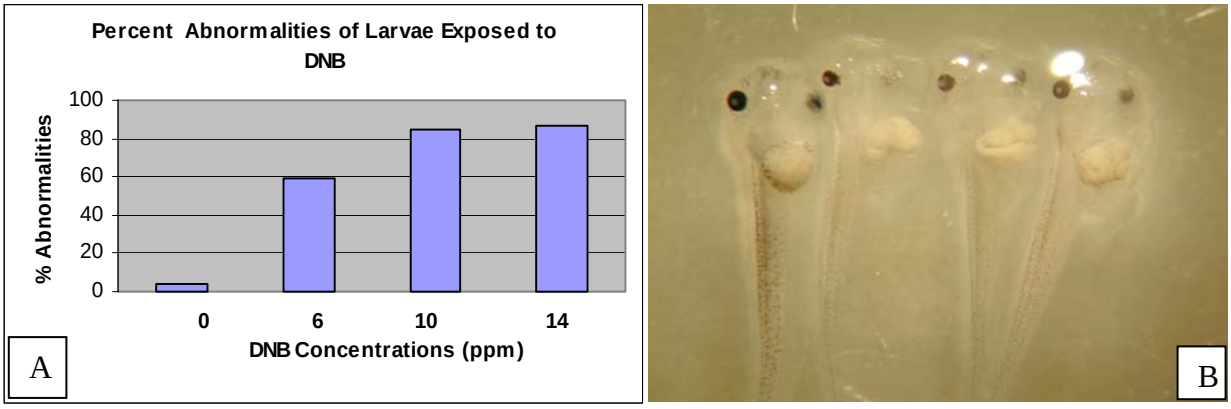
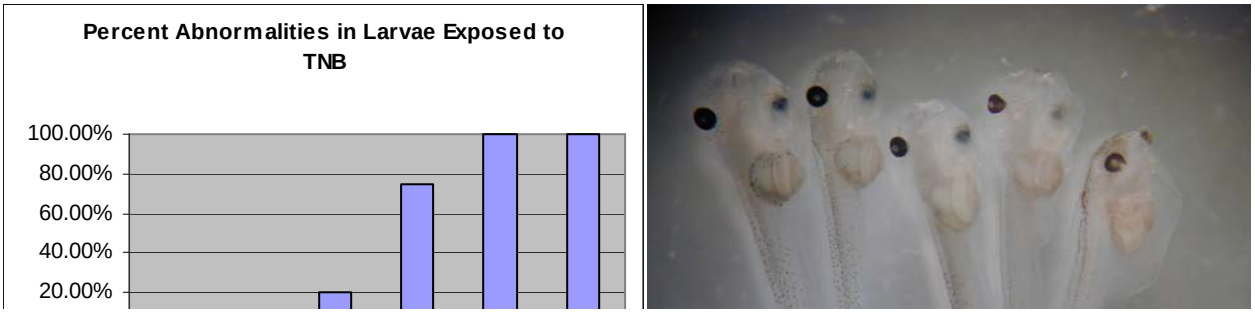


Figure 1. Showing a) the percent abnormality observed in *Xenopus laevis* larvae exposed to DNB for 96 hr; b) representative picture of abnormalities observed.

Exposure to DNB, at concentrations ranging from 6ppm to 26ppm, caused toxic insults to *Xenopus laevis* larvae. These responses were observed as deformities at the 3 lower concentrations of DNB and lethality at the higher concentrations. Deformities observed were scoliosis and varying degrees of edema in the abdominal and optic regions. The percentage of larva with edema in the control, 6ppm, 10ppm, and 14ppm groups were 4.0, 58.7, 85.1 and 86.3, respectively. A mortality rate of 100% was observed in larvae exposed to a concentration of 18ppm DNB or greater.

Dinitrobenzene did not affect hatch rate, snout-vent length, or stage of development. The hatch rate was 100% for all groups except those exposed to 10ppm in which a hatch rate of 96% was observed. Snout-vent length ranged from 7.5mm for 26ppm larva to 7.8mm for larvae in the control dose group.

Experiment II: Exposure to TNB



A

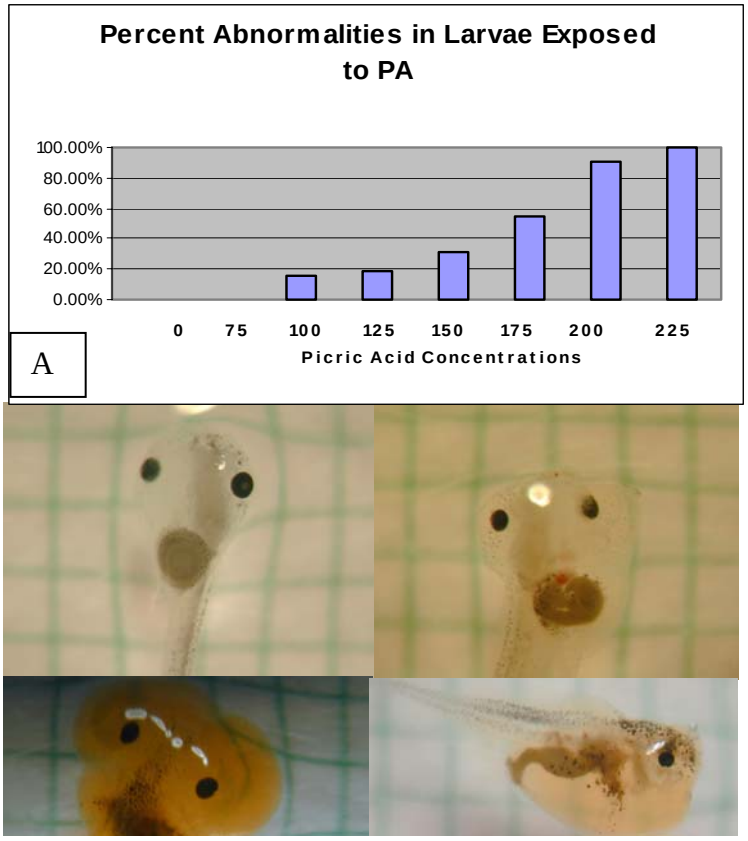
Figure 2. Showing a) the percent abnormality observed in *Xenopus laevis* larvae exposed to TNB for 96 hr; b) representative picture of abnormalities observed.

B

Exposure to TNB, at concentrations ranging from 0.5ppm to 3.5ppm, caused toxic insults in *Xenopus laevis* larvae. Deformities were observed at the 3 lower concentrations and lethality at the higher concentrations. Deformities observed were scoliosis and varying degrees of edema in the abdominal and optic regions. Edema in larvae exposed to control, 0.5ppm, 1ppm, 1.5ppm, 2ppm and 2.5ppm groups were 6.4, 8.2, 4.3, 2.1 and 36.2 percent respectively. A mortality rate of 100% was observed in the 2ppm, 3ppm and 3.5ppm exposure groups.

Trinitrobenzene affected hatch rate only at the 3.5ppm exposure level, where only 48% of the eggs hatched. Snout-vent length and mean N-F stage of development were not affected by treatment. Mean snout-vent length ranged from 6.4mm to 7.8mm while the N-F developmental staged averaged approximately 48 in the treated groups and the control.

Experiment III: Exposure to PA



B

Figure 3. Showing a) the percent abnormality observed in *Xenopus laevis* larvae exposed to PA for 96 hr; b) representative picture of abnormalities observed.

Toxic effects in response to PA-exposure, at concentrations ranging from 75ppm to 225ppm were observed as deformities and lethality in *Xenopus laevis* larvae. A biphasic response to PA-exposure was seen in these parameters. Apparent deformities observed were scoliosis and varying degrees of edema. The highest mortality rate at 64% was observed in larvae exposed to 225ppm PA.

Hatch rate, snout-vent length and mean N-F stage of development were not affected by treatment. Mean snout-vent length ranged from 6.4mm to 7.8mm while the N-F developmental staged averaged approximately 48 in the treated groups and the control.

Experiment IV: *Rana catesbeiana* Exposures

Data for the range finding studies are as shown in the tables below. These concentrations were selected based on toxicity data in other studies. The data is summarized in the following tables below.

Table 1. Effects to TNT and RDX on American bullfrog larval development

TNT				RDX			
CONC	HATCHED	SURVIVED	MORTALITY	CONC	HATCHED	SURVIVED	MORTALITY
80	30	0	100.00%	46	20	20	0.00%
40	30	0	100.00%	23	10	10	0.00%
20	29	0	100.00%	11.5	20	19	5.00%
10	30	27	10.00%	5.75	20	19	5.00%
5	30	26	13.33%	2.88	20	19	5.00%
2.5	30	28	6.67%	1.88	20	18	10.00%
0	20	20	0.00%	0	20	19	5.00%

Table 2. Effects to HMX and 4-ADNT on American bullfrog larval development

HMX				4-ADNT			
CONC	HATCHED	SURVIVED	MORTALITY	CONC	HATCHED	SURVIVED	MORTALITY
6	20	18	10.00%	10	20	20	0.00%
3	20	17	15.00%	8	20	20	0.00%
1.5	20	17	15.00%	6	20	20	0.00%
0.75	20	16	20.00%	4	20	20	0.00%

0.375	20	18	10.00%	2	20	20	0.00%
0.188	20	17	15.00%	1	20	20	0.00%
0	20	19	5.00%	0	20	20	0.00%

Table 3. Effects to 2-ADNTon American bullfrog larval development

2-ADNT

CONC	HATCHED	SURVIVED	MORTALITY
10	20	20	0.00%
8	20	20	0.00%
6	20	20	0.00%
4	20	20	0.00%
2	20	20	0.00%
1	20	20	0.00%
0	20	20	0.00%

16.0 DISCUSSION:

This study was designed to determine the acute effects of PA, TNB, and DNB, individually at concentrations in ppm, on developing *Xenopus laevis* larva. The 96hr LC50 values were calculated to be 284 ppm, 12.1 ppm and 2.0 ppm, respectively.

Exposure resulted in a concentration-dependent increase in frequency and severity of abnormalities. The main abnormalities that were observed in treated larvae were blisters, severe optic and thoracic edema, dorsal and lateral flexure of the tail, enlarged intestine, and incomplete coiling and lateral displacement of the intestine. Based on these observations, it is inconclusive whether these chemicals are teratogenic in this species. No apparent dysmorphogenesis was seen in any larva. The incomplete coiling and distended gastrointestinal tract observed are likely due to edema as these conditions were seen mainly in larvae that were severely edematous in the abdominal region. These abnormalities were primarily evident after the second day of exposure.

Neither hatch rate nor hatch date was affected by exposure to any of the chemicals at the concentrations used in this study. Similarly, snout-vent length was not affected and the NF stage of development was not delayed in treated larvae compared to control ones. After 96h of observation, NF stage 48 was the most frequently observed stage of larval development in the treatment and control groups. These results suggest that PA, TNB, and DNB at the concentrations used in this study appear to induce developmental toxicity to *Xenopus laevis* larvae during the early stages of development. These toxic effects seen in response to exposure to these chemicals appear to perturb biochemical homeostasis that might be related to critical regulatory pathways in the early developmental stage.

Anuran development is divided into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Dodd and Dodd, 1976; Etkin, 1964; 1968). During premetamorphosis, which is the period of embryonic and early larval stage, organogenesis and some advanced morphological changes such as hind limb bud development occurs without the influence of thyroid hormone. The prometamorphosis is

marked with more specific morphogenesis, such as differentiation of the toes and elongation of the hind limbs. The final period which is metamorphic climax, is associated with metamorphosis, including tail resorption and tail forelimb development. Thus, to determine the advanced stage of developmental toxicity of these chemicals it would be necessary to investigate exposure over these periods to stage NF-66.

The results from this study suggest that these chemicals are potentially hazardous to *Xenopus laevis* species, especially TNB and DNB with such low LC50s. The effects of these contaminants on native amphibian species that would be found at DoD contaminated sites, however; requires characterization and evaluation in such species (Theodorakis, 2004).

17.0 STUDY RECORDS AND ARCHIVE:

Study records will be maintained at The Institute of Environmental and Human Health Archive for a minimum of one year from study completion date.

18.0 REFERENCES:

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TITLE:	Bioavailability of RDX in aquatic ecosystems
STUDY NUMBER:	ZEB-06-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
ANIMAL TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
RESEARCH INITIATION:	January 2006
RESEARCH COMPLETION:	December 2006

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

_____ _____

Reynaldo Patiño Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

_____ _____

Brian Birdwell Date
Quality Assurance Manager

1. Descriptive Study Title:
Bioavailability of RDX in aquatic ecosystems

2. Study Number:
ZEB-06-01

3. Sponsor:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. Testing Facility Name and Address:
Texas Cooperative Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. Proposed Experiment Start and Termination Dates:

Start date:	January 2006
Termination Dates:	December 2006

6. Key Personnel:
Reynaldo Patiño, Testing Facility Management
Sandeep Mukhi, Study Director
George Cobb, Analytical Chemist
Brian Birdwell, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. Study Objectives/Purpose:
The objective of this study is to examine the role of aquatic flora (aquatic plants; Vallisneria) and fauna (fish; zebrafish) in determining the availability of RDX or its metabolites to the various biological components of the aquatic ecosystem.

8. Study Summary
The most notable finding of this study is that the concentrations of RDX in the water were lower at the completion of the exposure (4 weeks) when fish were present in the aquaria than when they were absent. A decline in RDX content was also observed in plant tissue at 4 weeks but not in the other compartments of the aquarium mesocosm. The significance of these observations is uncertain but it could be associated with inorganic nitrogen-dependent microbial fauna that were likely present in aquaria with fish.

The levels of MNX were generally low in all compartments (water, fish, plant, and substrate). Most compartments of the aquarium mesocosms analyzed in the present study were largely aerobic with only a small compartment, the plant substrate, being possibly

anaerobic because of its lack of water circulation. Since MNX is produced mostly by the anaerobic metabolism of RDX it is not surprising that the levels of MNX were generally low in all compartments and that the highest levels were found in the plant substrate.

9. Test Materials:

Test Chemical name: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)
CAS number: 121-82-4
Purity: 99%
Stability: The RDX solution is stable at least for 1 week in aquarium condition
Source: Accurate Energetics, (McEwen, TN, USA)

Reference Chemical name: R/O Right ™
CAS number: Not applicable
Characterization: An artificial salt mixture which provides natural water chemistry for the conversion of reverse osmosis, distilled or deionized water for fresh water fish.
Purity: Certificate of analysis or analytical testing will indicate purity.
Stability: Stable
Source: Aquatic Ecosystems, Inc.

Reference Chemical name: Ultra pure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.
CAS Number: Not applicable
Characterization: Water quality will be tested by chemical analysis and pH will be monitored regularly.
Purity: Ultra pure
Stability: Stable
Source: Steam plant deionized water that has been run through a carbon filter and a de-ionizer to convert it to ultra pure water. R/O Right ™ (24g / 100L) will be added to this water.

10. Justification of Test System:

The cyclic nitramine, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is widely used as an explosive in commercial and military operations. It is believed that the large-scale manufacture, use, and improper disposal of RDX have led to contamination of soil and ground water by this compound and its metabolites (Haas et al. 1990, Sunahara et al. 1998). RDX in natural soil environments is metabolized to other compounds (Sheremata et al. 2001). RDX metabolites may include hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Much of the waste generated through the use of explosive compounds finds its way into the aquatic ecosystem. In topsoil, nitroso metabolites of RDX (MNX, DNX and TNX) accumulate in the aqueous phase as the parent compound breaks down (Sheremata et al., 2001). In marine sediment, MNX is also an important metabolite of RDX (Zhao et al., 2004). Soil and sediment-based metabolism of RDX is presumably mediated by microbes. Plant cells can also transform

RDX to nitroso derivatives by light-independent processes (Van Aken et al., 2004). In previous studies, we have observed that MNX is a prominent metabolite of RDX in zebrafish tissues although the source of this metabolite (directly from water or from metabolism by tissue) is unknown. We are unaware of any literature indicating that RDX can be metabolized to nitroso compounds in animal tissues. Therefore, the MNX we found in zebrafish tissues may have originated from microbial metabolism of RDX in water of "sediment" (microbial populations in tank biofilters). A better understanding of this phenomenon is important for risk assessment of RDX exposures, and possibly also for application in RDX decontamination/detoxification technologies.

In previous studies (DoD-SERDP, Phase VI-VII), we examined the acute (lethal) and sublethal toxicity of RDX on the growth and reproduction in zebrafish (*Danio rerio*). The model organism for the present study continues to be zebrafish. This fish is frequently used in biomedical research and has the advantage of the existence of a wealth of information concerning its genetics and developmental biology. Also, the zebrafish genome sequencing project and the availability of commercial cDNA microarrays allow for the convenient application of current tools in molecular biology to this species. Further, zebrafish are easy and economical to maintain in the laboratory, and we have prior expertise and experience using this species for toxicological research.

The plant chosen for this study is tape grass (*Vallisneria americana*). This plant is a submerged plant species native to North America and, therefore, relevant from ecological point of view. *Vallisneria* is commercially available through aquarium fish outlets.

11. Test Animals and Plants:

Species: *Danio rerio*, Zebrafish
Strain: Wild type
Age: Adult
Number: 30 (5 in each tank x 6 tanks)
Source: Local vendor

Species: *Vallisneria americana*
Strain: Wild type
Age: NA
Mass: 30 g (approx).
Source: Local vendor

12. Procedure for Identifying the Test System:

The experimental units (aquarium) were labeled with the study protocol number, ACUC number, chemical and its concentration, and the contact person name on it. There were four replicates for each treatment and each treatment was color coded for easy visual identification.

13. Experimental Design Including Bias Control:

To understand the effect of aquatic flora and fauna on the availability of RDX and its metabolites in aquatic systems, the following experimental design was applied using 40-liter aquaria as experimental units:

1. Tanks with water only
2. Tanks with water and fish
3. Tanks with water and aquatic plants
4. Tanks with water, aquatic plants and fish

RDX was added to the tanks to a final concentration of 3 ppm on the same day that the appropriate ecosystem component were placed together (1-4 above). The same initial number of fish (5 fish in each tank) and plant biomass (approx. 30 g) were added to the appropriate tanks. All treatments will be conducted in triplicate. At 4 weeks after the onset of the RDX exposure, concentrations of RDX and its main metabolite (MNX) were measured in sub-samples of water, aquatic plants, fish and biofilters, as appropriate, from each tank. Fish and plant biomass were determined in the appropriate tank.

14. Methods:

14.1 Test System acquisition, quarantine, and acclimation

Adult zebrafish and aquatic plants were purchased from a local vendor and acclimated to the laboratory prior to use in the test systems. Namely, zebrafish were allowed to acclimate to for a period of one week to standard temperature, pH and light/dark cycle (photoperiod). The optimal values for zebrafish are pH 6.0-8.5, 26-30°C, 12/12 light/dark cycle.

14.2 Test Condition Establishment

A static method of exposure was used, and the duration of exposure was 4 weeks. For each treatment, four aquaria (replicates) were prepared. At the onset of the exposure period, all tanks were spiked with RDX to achieve the desired concentration of 3 ppm. Four hand-made biofilters were placed in each tank, and they were supplied with air for maintaining water current for proper filtration. Aquatic plants (approximately 30 g) were placed in the appropriate aquaria with the help of glass-bead as substratum. Five zebrafish were randomly allocated into the appropriate tanks on the starting day of the experimentation. Water quality was maintained according to zebrafish husbandry TCFWRU SOP AQ-1-01 (pH 6.0-8.5, 26-30^{O}C, 12/12 light/dark cycle), in ultra pure water with salts. (For additional details on zebrafish husbandry see TCFWRU SOP AQ-1-01.)

14.3 Test Material Application

At the beginning of the experiment each tank received RDX slurry to obtain a desired concentration of 3 ppm. There was not any water exchange during the entire period of the experiment (4 weeks). The tanks were topped off with deionized water every week to

compensate for evaporative losses. RDX and MNX concentrations in the water were monitored in sub samples (5 ml) collected once weekly.

Rates/concentrations: 3 ppm

Frequency: Once at the beginning of the exposure period

Route/Method of Application: Method of application was by immersion. Route of exposure was dermal, oral, and respiratory for fish and through root and leaf surface for the plants (as the chemical will be in water).

Justification for Exposure Route: Exposure by rearing in water is most appropriate as run off and sewage effluents enter water systems such as lakes and streams. Because fish respire, ingest, and are dermally exposed to the water in which they live, this situation is considered environmentally relevant. A similar situation applies to aquatic plants.

Exposure Verification: Water samples were collected and verified for the presence of RDX in each treatment tank.

14.4 Test System Observation

The test system was monitored daily. Fish mortality, if any, was recorded. The general health of the fish and plants was monitored daily.

14.5 Animal Euthanasia and Sample Collection

At the end of the exposure, fish were euthanized by immersion in 1.0 g/L buffered MS-222 solution (TCFWRU SOP AQ-1-03).

14.6 Endpoint Analysis

RDX and MNX concentrations in water, biofilters, plant tissue, and whole-body zebrafish homogenates were measured after 4 weeks of exposure

15.0 Results:

The concentration of RDX in water from all treatments was slightly higher than the nominal concentration of 3 ppm (Figure 1). At the end of the exposure (4 weeks), levels of RDX in those aquaria containing fish were lower than in the aquaria without fish (Figure 1 and 2). MNX was not detected in the water samples.

Neither RDX nor MNX levels in fish were affected by the presence or absence of plants, although MNX levels in fish tissue were consistently undetectable in the presence of plants (Figure 3). Curiously, the levels of RDX in plants were lower in the presence of fish than in their absence (Figure 4).

The concentrations of RDX and MNX in the aquarium biofilters (Figure 5) and the plant substrates (Figure 6) were not affected by treatment.

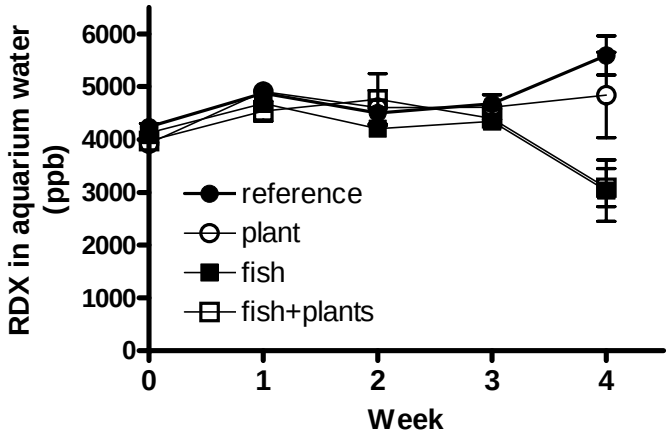

Figure 1. RDX in aquarium water during the exposure.
Water samples were collected immediately upon start of the exposure (Week 0) and at the end of each exposure week (Week 1-4). An initial 2-way ANOVA (repeated measures) indicated treatment, time and interaction effects ($p<0.05$). Subsequent 1-way ANOVA at each sampling time indicated treatment effects only at the end of Week 4 (see figure 2).

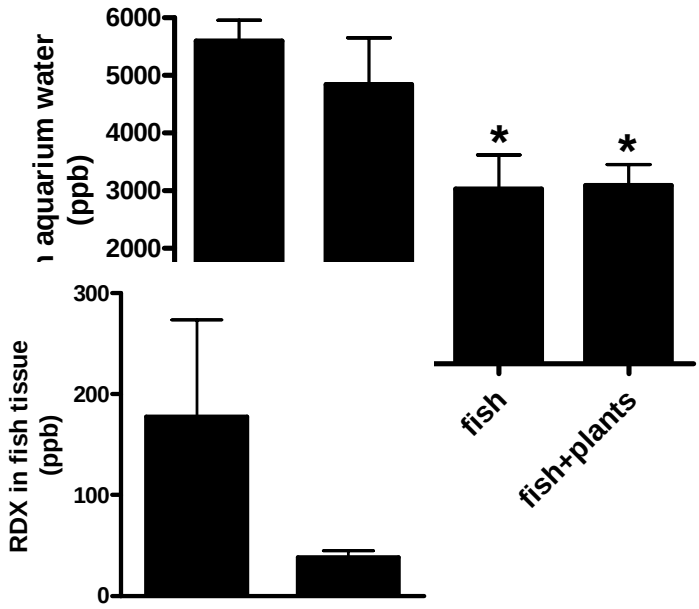

Figure 2. RDX in aquarium water at the end of the exposure.
Water samples collected at the end of Week 4. Asterisks indicate significant difference from reference treatment (1-way ANOVA and Dunnett's Multiple Comparison Test, $p<0.05$).

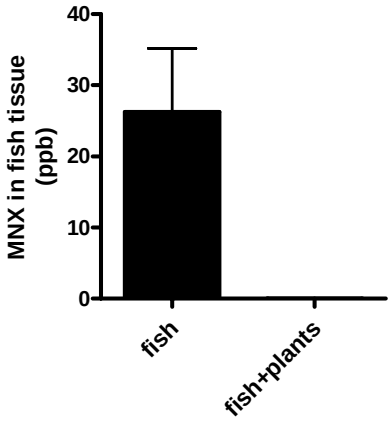

Figure 3. RDX and MNX in fish tissue.
The concentration of RDX in fish tissue did not differ when plants were absent (fish) or present (fish+plants) in the aquaria (Student's *t*-test on log-transformed data, $p>0.05$), although there was a trend for lower values in the presence of plants. Levels of MNX were not detectable in fish tissue

when plants were present in the aquaria, but the non-parametric statistical analysis used indicated no statistically significant difference (Wilcoxon Signed Rank Test, $p>0.05$).

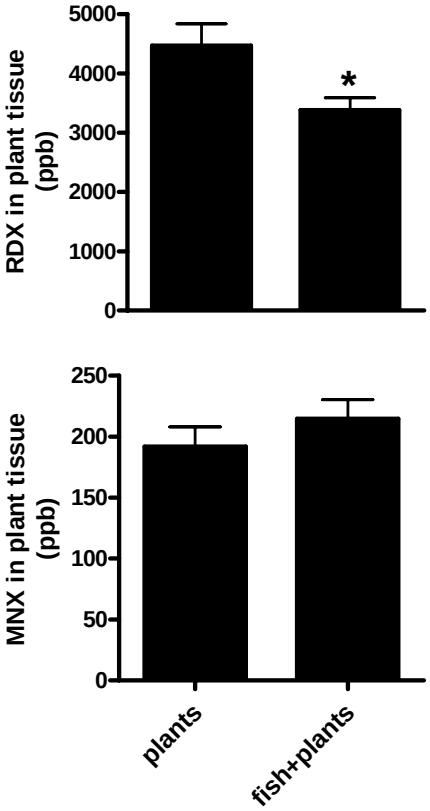

Figure 4. RDX and MNX in plant tissue.
The levels of RDX in plants were lower when fish were present in the tank than when fish were absent (Student's *t*-test, $p<0.05$). The levels of MNX did not differ between the groups (Student's *t*-test, $p>0.05$).

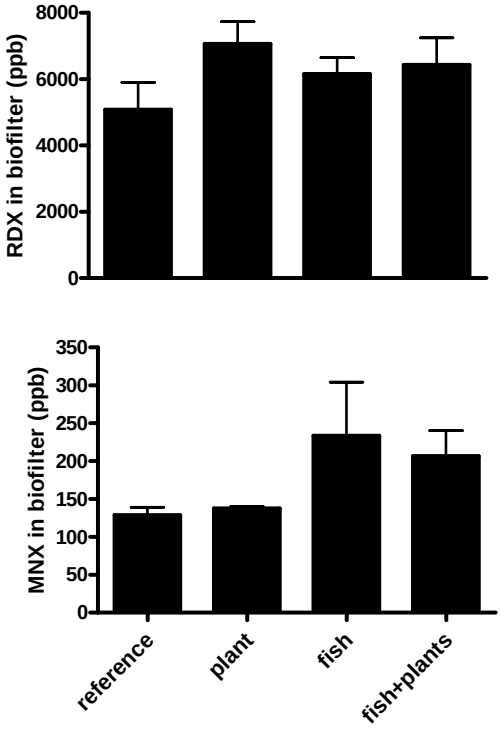

Figure 5. RDX and MNX concentrations in aquarium biofilters.
Water constantly circulated through the in-tank biofilters during the exposure period. Biofilters consisted of packed glass wool and contained considerable waste and debris at the end of the exposure. Because of the constant water circulation, they are likely to have been populated by aerobic microbes. Mean concentrations did not differ among treatments for both compounds (1-way ANOVA, $p>0.05$; log-transformed values were used for MNX analysis).

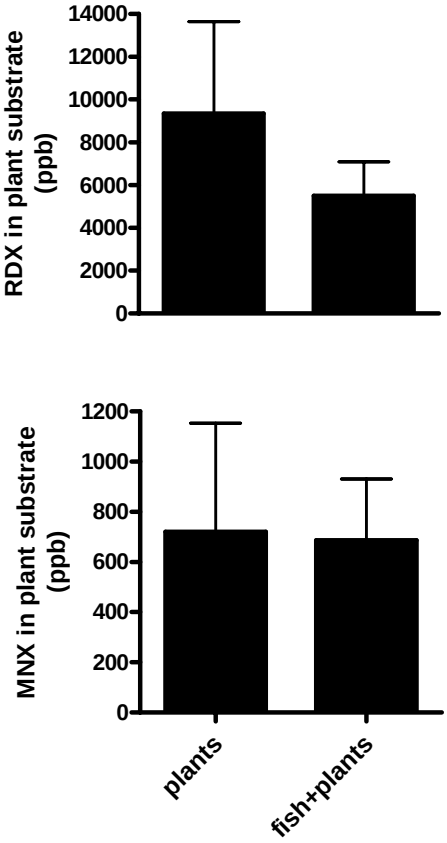

Figure 6. RDX and MNX concentrations in plant substrate.
Aquatic plants were planted in small beakers containing packed wool (substrate). Water did not circulate through the substrates and their conditions are likely to have been hypoxic. Mean concentrations of either RDX or MNX did not differ among treatments (Student's *t*-test, $P>0.05$).

16.0 Discussion:

The most notable finding of this study is that the concentrations of RDX in the water were lower at the end of the exposure period (4 weeks) when fish were present in the aquaria than when they were absent. The significance of this observation is uncertain, but it could be associated with inorganic nitrogen-dependent microbial fauna that were likely present in the aquaria with fish. Namely, the aquaria where fish were present most likely developed a population of ammonia-dependent nitrification bacteria. Ammonia is the main product of protein metabolism by the fish and during the typical cycle of a new fish aquarium, it takes several weeks for this microbial fauna to become established. The ammonia is first converted into nitrite and then into nitrate by two different microbial groups. The general timeline for the "cycling" of an aquarium is consistent with the timing of the first detectable decline in water RDX concentrations in the present study: 4 weeks from the start of the exposure. A decline in RDX content was also observed in plant tissue at 4 weeks but not in the other compartments of the aquarium mesocosm.

Most compartments of the aquarium mesocosms analyzed in the present study were largely aerobic with only a small compartment, the plant substrate, being possibly anaerobic because of its lack of water circulation. Since MNX is produced mostly by the anaerobic metabolism of RDX (Wani et al., 2003), it is not surprising that the levels of MNX were generally low in all compartments and that the highest levels were found in the plant substrate.

Although anaerobic metabolism of RDX is believed to occur more readily than aerobic metabolism, RDX can be metabolized under aerobic conditions (Wani et al., 2003). In fact, certain microbes from RDX-contaminated soil are able to degrade RDX more rapidly in the presence of oxygen (Waisner et al., 2002). It is not known whether ammonia-dependent aerobic microbes typical of fish aquaria can metabolize RDX. Also, assuming that these microbes do metabolize RDX, it would be unclear why in the absence of fish, RDX alone would not suffice to provide the nitrogen source to support their growth, thus, also leading to a decline in water RDX concentrations. We suggest that these questions deserve further consideration.

17.0 Study Records and Archive

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after the completion date of the study.

18.0 References

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